

Henri Bourg
Amaury Lisle
Editors

BIOMATERIALS

Developments and Applications

Advances in Biology and Medicine Series

NOVA

ADVANCES IN BIOLOGY AND MEDICINE SERIES

BIOMATERIALS DEVELOPMENTS AND APPLICATIONS

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ADVANCES IN BIOLOGY AND MEDICINE SERIES

BIOMATERIALS DEVELOPMENTS AND APPLICATIONS

**HENRI BOURG
AND
AMAURY LISLE
EDITORS**

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PREFACE

Despite the huge impact that biomaterials have had on patients' quality of life, improvements in device performance and the development of alternatives to augment available therapies are continuously being sought. This book explores the development and application of biomaterials over the past 25 years, examining the current clinical demand, the scientific rationale, and the technical challenges to be overcome. Furthermore, this book introduces the blood components involved in hemostasis and thrombosis, followed by the common biomaterials applied in blood-contacting devices. The complications induced by the interactions between blood and biomaterials is also briefly addressed, as well as the commonly used techniques for improving biomaterials' hemocompatibility. In addition, the authors give some valuable insight into calcium orthophosphate cements and concretes, as excellent biomaterials suitable for both dental and bone grafting application. Despite the growth of potential hydrogel based therapies, the route to market for new products is constrained by the need to develop an appropriate regulatory and metrological framework under which they will be assessed. This book outlines the role that metrology has to play in the regulation of emerging hydrogel-based therapies and detail possible strategies for assessing their safety and effectiveness. Other chapters examine the surface of CoCrMo alloy prior and after exposure into different solutions simulating oral conditions, recent research on delivery of gold nanoparticles inside the cells and their potential applications in nanomedicine, and the widely used biomedical applications for hydrogels.

Chapter 1 - In this book, the state-of-the-art of calcium orthophosphate-based biocomposites and hybrid biomaterials suitable for biomedical applications is presented. This subject belongs to a rapidly expanding area of science and research because these types of biomaterials offer many significant and exciting possibilities for hard tissue regeneration. Through the successful combinations of the desired properties of matrix materials with those of fillers (in such systems, calcium orthophosphates might play either role), innovative bone graft biomaterials can be designed. The book starts with an introduction to locate the reader. Further, general information on composites and hybrid materials, including a brief description of their major constituents are presented. Various types of calcium orthophosphate-based bone-analogue biocomposites and hybrid biomaterials those are either already in use or being investigated for various biomedical applications are then extensively discussed. Many different formulations in terms of the material constituents, fabrication technologies, structural and bioactive properties, as well as both in vitro and in vivo characteristics have been already proposed. Among the others, the nano-structurally controlled biocomposites,

those with nano-sized calcium orthophosphates, biomimetically-fabricated formulations with collagen, chitin and/or gelatin, as well as various functionally graded structures seem to be the most promising candidates for clinical applications. The specific advantages of using calcium orthophosphate-based biocomposites and hybrid biomaterials in the selected applications are highlighted. As the way from a laboratory to a hospital is a long one and the prospective biomedical candidates have to meet many different necessities, the critical issues and scientific challenges that require further research and development, have been examined, as well.

Chapter 2 - In early 1980-s, researchers discovered self-setting calcium orthophosphate cements, which are a bioactive and biodegradable grafting material in the form of a powder and a liquid. Both phases after mixing form a viscous paste that after being implanted sets and hardens within the body as either a non-stoichiometric calcium deficient hydroxyapatite (CDHA) or brushite, sometimes blended with unreacted particles and other phases. As both CDHA and brushite are remarkably biocompatible and bioresorbable (therefore, *in vivo* they can be replaced with a newly forming bone), calcium orthophosphate cements represent a good correction technique of non-weight-bearing bone fractures or defects and appear to be very promising materials for bone grafting applications. Besides, these cements possess an excellent osteoconductivity, molding capabilities and easy manipulation. Nearly perfect adaptation to the tissue surfaces in bone defects and a gradual bioresorption followed by new bone formation are additional distinctive advantages of calcium orthophosphate cements. Furthermore, reinforced cement formulations are available, which in a certain sense might be described as calcium orthophosphate concretes. The concepts established by calcium orthophosphate cement pioneers in the early 1980-s were used as a platform to initiate a new generation of bone substitute materials for commercialization. Since then, advances have been made in the composition, performance and manufacturing; several beneficial formulations have already been introduced as a result. Many other compositions are in experimental stages. In this review, an insight into calcium orthophosphate cements and concretes, as excellent biomaterials suitable for both dental and bone grafting application, has been provided.

Chapter 3 - The range of materials used for biomedical applications is very broad. This means that the demands on their properties are very diverse depending on various medical areas and applications. Moreover, it is often necessary to have available materials with the possibility to set the required parameters very precisely in very wide ranges. Because of the similar mechanical behaviour of hydrogels with that of living tissues and their good compatibility and ability of hydrogels to swell in water, the hydrogels are often used in biomedical applications.

Hydrogel polymers are natural or synthetic hydrophilic crosslinked polymers favourably interacting with water. The swelling degree (the water content in equilibrium-swollen gel) is a function of polymer hydrophilicity and the degree of crosslinking and has influence on a number of physical and chemical properties (e.g., refractive index, transport properties). In biomedical applications, the physical structure and morphology of the hydrogels is adjusted to the targeted performance and both homogenous and heterogeneous materials with porous, nanofiber, or nanoparticle structures are used.

One of the oldest and still widely used biomedical applications of hydrogels is the contact lens. Hydrogels are successfully applied as a variety of implants in surgery, ophthalmology, otorhinolaryngology, neurology, urology, gynaecology etc. In addition, hydrogels are used to

cover wounds, burns, trophic defects, or as a two-dimensional supports for cultivation and potential transplantation of cells or as three-dimensional scaffolds for tissue engineering and cell therapy.

In some applications, particularly, in tissue engineering, the biodegradable materials (hydrogels) are used. The advantage is that after the fulfilment of their tasks (e.g., proliferation of cell culture), they disintegrate (hydrolytically or enzymatically) and, subsequently, they are eliminated from the organism. Controlled transport and release of drugs is a separate biomedical area.

Hydrogels for biomedical applications can be generally classified in different categories depending on their interaction with the living tissue. The hydrogels are used either in direct contact with tissues (implants, injection needle, infusion sets, bandages, suture materials, carrier of drugs, cell or tissue culture) or in indirect contact with tissues (orthesis, medical devices, air filters, sanitary supplies, etc.).

Chapter 4 - The widespread use of biomaterials in medicine and dentistry is a relatively new phenomenon dating back to the 1950's yet, today, an estimated 20 million individuals have an implanted medical device.

Despite the huge impact that biomaterials have had on patients' quality of life, improvements in device performance and the development of alternatives to augment available therapies are continuously being sought. Clinical demand, advances in molecular and cell biology and the increased understanding of the role of the tissue-material interface on clinical performance has led to a metamorphosis of the biomaterials' field over the past 25 years. This has resulted in a change in the nature of biomedical devices from being biologically passive to actively integrated.

This chapter explores the development and application of biomaterials over the past 25 years, examining the current clinical demand, the scientific rationale, and the technical challenges to be overcome. As biomaterials are applied in reconstructive surgery and tissue regenerative therapies, these areas are explored with specific examples of recent developments and current research activity used to illustrate the changing perspectives.

Chapter 5 - All blood-contacting medical devices in use today are subjected to some degree poorer blood compatibility than the native artery. Hemostatic mechanism, arresting bleeding from injured blood vessels, induces platelet adhesion and activation onto artificial biomaterials, which leads to undesirable outcomes such as blood clotting at the site of the implant, continual shedding of thrombi, and depletion of platelets from the blood stream. Such complications have hampered the clinical success of blood contacting devices, limiting the patency of small-diameter vascular grafts and making necessary the use of anticoagulants in patients undergoing extracorporeal bypass or synthetic heart valve implantation. Therefore, development of non-thrombogenic biomaterials is in great need for blood contacting devices. The current approaches mainly focus on surface modifications with biological anticoagulants such as heparin, or anti-fouling molecules like poly(ethylene oxide). In this review, the authors will first introduce the blood components involved in hemostasis and thrombosis, followed by the common biomaterials applied in blood-contacting devices. Next, the complications induced by the interactions between blood and biomaterials will be briefly addressed. Finally, the commonly used techniques for improving biomaterials' hemocompatibility will be expatiated.

Chapter 6 - A number of biomaterials, organic or synthetic, are used in the various fields of medical or dentistry. Among them are the suture materials, osteosynthesis plates and

screws, endosseous implants, bone substitutes, endodontic, restorative, prosthetic materials, and others. All of them are put in contact with biological tissues, deeply or superficially and most of them remain for lifetime. For this reason, all these materials should be carefully analyzed by means of biocompatibility tests, in order to evaluate materials' biological behavior. In this regard, the goal of this chapter is to present the biocompatibility of some biomaterials such as bioactive bone substitutes (ceramics, bioactive glasses and polymers) titanium endosseous implants, metal and polymeric osteosynthesis devices, restorative and endodontic materials based on the author's current research with the field.

Chapter 7 - In an attempt to further the anticaries benefits afforded by fluoride, the authors have developed a unique functionalized tricalcium phosphate (fTCP) and explored its remineralization potential in several dental models. fTCP was found to be stable in NaF aqueous solutions of 500, 950, and 5000 ppm F, and is the first form of bioavailable calcium that is stable with fluoride in an aqueous and single-compartment environment. Using an *in vitro* pH cycling regimen emulating actual events transpiring in the oral cavity, the authors demonstrated that the baseline remineralization of softened enamel with topical solutions of 500, 950, and 5000 ppm F can be substantially improved when combined with fTCP. Using both Vickers and Knoop microhardness measurements, the reversal of white-spot lesions was attributed to both surface and bulk remineralization effects with fTCP providing statistical improvement over the base fluoride solution. However, in a single-step dental model, fTCP was found to limit fluoride uptake. This result was inconsistent with multiple-step regimens and previously reported work, but furthers the author's understanding of the influence fTCP has on enamel. Because fTCP does not compromise fluoride bioavailability and is not limited to boosting remineralization through elevating fluoride uptake alone, the authors explored the role fTCP has with enamel tissue and fluoride. Based on the author's results, the authors conclude fTCP intertwines synergistically with both fluoride and the enamel tissue in a sophisticated manner that produces superior remineralization of the subsurface lesion relative to fluoride alone.

Chapter 8 - Traditionally hydrogels have been widely used in medicine for sight correction, as coupling agents, barrier coatings and vehicles for drug delivery. Such materials are relatively simple in terms of their design and performance. This situation is rapidly changing as hydrogels begin to find a use in emerging healthcare solutions particularly in regenerative medicine and advanced drug delivery. Hydrogels capable of stimuli responsiveness, self-assembly, and molecular selectivity can now be produced for therapeutic applications owing to advances in hydrogel synthesis.

Despite the growth of potential hydrogel based therapies, the route to market for new products is constrained by the need to develop an appropriate regulatory and metrological framework under which they will be assessed. This chapter will outline the role that metrology has to play in the regulation of emerging hydrogel-based therapies and detail possible strategies for assessing their safety and effectiveness. It will be concluded that there is a need to improve current metrology in relation to hydrogels in order to expedite their translation into clinical practice.

Chapter 9 - Objectives: The prevailing biological conditions in the human oral cavity induce the release of trace element (TE) metal cations from the dental casting alloys. The authors studied the release of metal ions (Al, Ag, Au, Ca, Cd, Co, Cr, Cu, Mg, Mo, Ni, Pd, Pt, Ti, and Zn) from three most common dental casting alloys, two base and one noble:

Co•Cr•Mo, Ni•Cr, and Au•Pt alloy into three buffered biological solutions simulating the composition and pH of respective saliva, acidity, and dental plaque.

Methods: The commercially available Co•Cr•Mo, Ni•Cr and Au•Pt alloy specimens were soaked in pH 6.0 phosphate buffer (*Saliva*), 3.5 pH phosphate buffer (*Acid*) and pH 3.5 mixture of lactic, formic and acetic acid (dentobacterial *Plaque*), and incubated at 37 °C for 1, 2, 3, 4, 5, 6, 7, 14, 21, and 30 days. Six samples (n = 6) of every solution were prepared for every time period. Inductively coupled plasma atomic emission spectroscopy was used for analysis of the released elements.

Results: The concentration of the trace elements (TE) released from soaked dental metal alloys in various solutions of different pH are expressed in $\mu\text{g}\cdot\text{L}^{-1}$ as Mean values (SD). Average detectable amounts of TE from the Co•Cr•Mo dental alloy were released into: *Plaque* Co 502 (412), *Acid* Cr 589 (84), *Plaque* Fe 180 (43), *Acid* Ni 80 (96), and *Acid* Zn 87 (56). The manufacturers did not indicate the presence of Fe, Ni, and Zn. Average detectable amounts of TE from the Ni•Cr dental alloy were released into: *Acid* Co 347 (394), *Saliva* Cr 397 (491), *Saliva* Fe 248 (257), *Saliva* Ni 542 (669), and *Plaque* Zn 96 (51). The manufacturers did not indicate the presence of Co and Zn. Average detectable amounts of TE from the Au•Pt dental alloy were released into: *Acid* Cr 895 (14), *Plaque* Cu 113 (79), *Plaque* Zn 207.4 (25.3), and *Acid* Fe 150 (43). The manufacturers did not indicate the presence of Cr and Fe. ANOVA revealed the significant effect of alloy composition, the respective buffer solution (*Saliva*, *Acid*, *Plaque*), time interval (*1 to 30 days*), and interaction on ion release ($P < 0.001$ for every compared parameter).

Conclusion: There was a great within the buffer solution variability in metal ion release from the same dental alloy indicating their inhomogeneity. The base metal alloys virtually released more ions than the Au•Pt alloy, however their effective surface area was larger than of noble alloy. The ion release from an alloy doesn't necessarily correlate with the abundance of the element in the alloy. Rather, there is a selective dissolution so that the elements that are present in alloys only in traces can be released from them in larger amounts. The concentration of the major essential TE of Cu, Fe and Zn released from base dental alloys was below the Recommended Dietary Allowances (RDA's), whereas the leaching concentrations of the allergenic Co, Cr, and Ni were substantial. Considerable amounts of Ni was released from Ni•Co dental alloy, whereas undeclared Co from the Ni•Cr alloy and undeclared Ni from the Co•Cr•Mo alloy may have both local and systemic, especially allergenic, adverse effects. The undeclared chromium from noble Au•Pt dental alloy appears to be responsible for the contact allergy thus far attributed to the gold.

Chapter 10 - Introduction: It has been well-documented that metal ions are released from all dental alloys, which may be of a clinical concern and may be a potential health problem. Corrosion behaviour in oral fluids is strongly influenced by microstructural characteristics and surface properties of dental alloy.

Aim of the Study: To examine the surface of CoCrMo alloy prior and after exposure into different solutions simulating oral conditions, as well as prior and after different mechanical polishing procedures.

Materials and Methods: The cast and electropolished specimens of CoCrMo alloy (EP) (Wironit®, extra hard) were examined by SEM with EDS, by optical microscope and by AFM prior and after immersion into two solutions simulating *saliva* and dentobacterial *plaque* for 30 days at 37 °C. The effect of polishing at the surface of CoCrMo alloys has also been studied. After electropolishing procedures (EP, 6 samples) another samples were further

mechanically polished using green rubber discs and afterwards a high shine polishing paste and a rotating black brush (BB, 6 samples), or using green rubber discs, a high shine polishing paste and a rotating deer leather brush (DL, 6 samples).

Results: The AFM analysis provided useful information about the surface morphology. AFM imaging revealed that the surface of EP specimens prior the contact with corroding solutions was characterized with at least 3-4 different phases: undulating surface corresponding to dendrites in the optical microscope images, clusters of nanocrystallites emerging from the undulating surface (islands of nanocrystallites) corresponding to the interdendritic phase in the optical microscope images and some high conical crystallites emerging from both, undulating surface and the islands of nanocrystallites. Also some very small grain-like crystallites were also observed from AFM images. The undulating surface was twice richer with Co than Cr, while in the interdendritic phase (clusters of nanocrystallites forming islands and emerging from the undulating surface) Co was almost equal to Cr, which was different from the declared alloy's composition. However, some crystallites were dissolved after corrosion, but some new phases (dense sprinkles of very small crystallites) appeared. The respective alloy peaks in the EDS spectrum revealed that these phases were a result of a corrosion - primarily of metal dissolution and salt formation. The presence of some elements from the corroding solution was registered at the alloy surface, as well. The effect of mechanical polishing was clearly evident at CoCrMo surface. The clusters of nanocrystallites forming islands (emerging from the undulated surface) were smoothened and/or thorn away by further mechanical polishing, while the islands' perimeter was unaltered. Mechanical polishing also produced scratches in both, BB and DL samples. The scratches were observed not only at the undulating surface, but also at the smoothed islands. The Z range (distance from the highest to the lowest peak) of EP samples revealed significantly higher values than that of BB and DL ($p < 0.01$). The highest RMS and the Ra values were also recorded in the EP samples, but not significantly different ($p > 0.05$), compared to the mechanically polished BB and DL samples.

Conclusions: AFM imaging revealed at least four different phases at the surface of CoCrMo electropolished specimens. The most dominant phases were: undulating surface and clusters of nanocrystallites emerging from the undulating surface forming islands, but some high conical crystallites and some small grain-like crystallites have also been observed. Mechanical polishing of EP samples smoothed islands of nanocrystallites, but also formed scratches at the surface, which were more prominent in DL than BB samples. During the corrosion process of EP samples probably metal salts (anions from corroding solutions) were binded at the surface as a new phase. Electrochemical behaviour of CoCrMo alloys is clearly affected by pH and composition of the fluids. It is obvious that some complex mechanisms involving reactions between ionic species are dominating the interfacial processes, which in turn affect the morphology and the composition of the phases formed.

Chapter 11 - Nanotechnology has enormous potential in biomedical research including disease diagnosis and therapy. Inorganic nanoparticles have been adopted for biomedical research due to their unique optical and physical properties. Compared to conventional materials, inorganic nanomaterials have several advantages such as simple preparative process and precise control over their shape, composition and size. A major aim of medicine is the early and precise diagnosis of clinical conditions, providing an efficient treatment without secondary effects. Nanoparticle-based technologies have demonstrated especially high potential for medical purposes, ranging from diagnosing diseases to providing novel

therapies. Among the metal nanoparticles, gold nanoparticles have proven to be powerful tools in various nanomedicinal applications because of their photo-optical properties and biocompatibility. However, the existing nanoparticle-based technologies must overcome several challenges, including selective nanoparticle delivery, potential toxicity, imaging of nanoparticles and therapeutic efficacy. This chapter describes a brief overview of the recent research on delivery of gold nanoparticles inside the cells and their potential applications in nanomedicine.

Chapter 12 - Damage to the Central Nervous System (CNS) from disease or injury remains a serious problem that affects millions of individuals worldwide. Regeneration across a lesion in the adult mammalian CNS is autonomically inhibited, thereby presenting substantial challenges for clinical treatment strategies. Current methods employed such as autografts, xenografts and allografts have limited success due to the complex nature of CNS injury pathophysiology. As a result, there is no effective treatment available to establish repair of damaged neurones within the CNS, with patients typically being administered a combination of drugs and rehabilitation in attempts to maintain and restore some functionality. Recent research has explored the potential of synthetic scaffolds to fabricate biomimetic regenerative microenvironments for nerves as an alternative treatment option. This commentary addresses the importance of scaffold design in enhancing the regeneration capacity of the adult CNS; focusing on past innovative strategies and current problems. Future trends towards fabricating scaffolds to achieve superior regenerative outcomes will also be highlighted.

Chapter 1

CALCIUM ORTHOPHOSPHATE-BASED BIOCOMPOSITES AND HYBRID BIOMATERIALS

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ABSTRACT

In this book, the state-of-the-art of calcium orthophosphate-based biocomposites and hybrid biomaterials suitable for biomedical applications is presented. This subject belongs to a rapidly expanding area of science and research because these types of biomaterials offer many significant and exciting possibilities for hard tissue regeneration. Through the successful combinations of the desired properties of matrix materials with those of fillers (in such systems, calcium orthophosphates might play either role), innovative bone graft biomaterials can be designed. The book starts with an introduction to locate the reader. Further, general information on composites and hybrid materials, including a brief description of their major constituents are presented. Various types of calcium orthophosphate-based bone-analogue biocomposites and hybrid biomaterials those are either already in use or being investigated for various biomedical applications are then extensively discussed. Many different formulations in terms of the material constituents, fabrication technologies, structural and bioactive properties, as well as both in vitro and in vivo characteristics have been already proposed. Among the others, the nano-structurally controlled biocomposites, those with nano-sized calcium orthophosphates, biomimetically-fabricated formulations with collagen, chitin and/or gelatin, as well as various functionally graded structures seem to be the most promising candidates for clinical applications. The specific advantages of using calcium orthophosphate-based biocomposites and hybrid biomaterials in the selected applications are highlighted. As the way from a laboratory to a hospital is a long one and the prospective biomedical candidates have to meet many different necessities, the critical issues and scientific challenges that require further research and development, have been examined, as well.

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INTRODUCTION

The fracture of bones due to various traumas or natural aging is a typical type of a tissue failure. An operative treatment frequently requires implantation of a temporary or a permanent prosthesis, which still is a challenge for orthopedic surgeons, especially in the cases of large bone defects. A fast aging of the population and serious drawbacks of natural bone grafts make the situation even worse; therefore, there is a high clinical demand for bone substitutes. Unfortunately, a medical application of xenografts (*e.g.*, bovine bone) is generally associated with potential viral infections. In addition, xenografts have a low osteogenicity, an increased immunogenicity and, usually, resorb more rapidly than autogenous bone. Similar limitations are also valid for human allografts (*i.e.*, tissue transplantation between individuals of the same species but of non-identical genetic composition), where the concerns about potential risks of transmitting tumor cells, a variety of bacterial and viral infections, as well as immunological and blood group incompatibility are even stronger [1-3]. Moreover, harvesting and conservation of allografts (exogenous bones) are additional limiting factors. Autografts (endogenous bones) are still the “golden standard” among any substitution materials because they are osteogenic, osteoinductive, osteoconductive, completely biocompatible, non-toxic and do not cause any immunological problems (non-allergic). They contain viable osteogenic cells, bone matrix proteins and support bone growth. Usually, autografts are well accepted by the body and rapidly integrated into the surrounding bone tissues. Due to these reasons, they are used routinely for a long period with good clinical results [3, 4]; however, it is fair to say on complication cases, those frequently happened in the past [5, 6]. Unfortunately, a limited number of donor sites restrict the quantity of autografts harvested from the iliac crest or other locations of the patient’s own body. Also, their medical application is always associated with additional traumas and scars resulting from the extraction of a donor tissue during a superfluous surgical operation, which requires further healing at the donation site and can involve long-term postoperative pain [1, 6-9]. Thus, any types of a biologically derived transplants appear to be imperfect solutions, mainly due to a restricted quantity of donor tissues, donor site morbidity, as well as potential risks of an immunological incompatibility and disease transfer [7, 9, 10]. In this light, manmade materials (alloplastic or synthetic bone grafts) stand out as a reasonable option because they are easily available, might be processed and modified to suit the specific needs of a given application [11, 12]. What’s more, there are no concerns about potential infections, immunological incompatibility, sterility and donor site morbidity. Therefore, investigations on artificial materials for bone tissue repair appear to be one of the key subjects in the field of biomaterials research for clinical applications [13].

Currently, there are several classes of synthetic bone grafting biomaterials for *in vivo* applications [14-17]. The examples include natural coral, coral-derived materials, bovine porous demineralized bone, human demineralized bone matrix, bioactive glasses, glass-ceramics and calcium orthophosphates [9]. All of these biomaterials are biocompatible and

osteoconductive, guiding bone tissue from the edges toward the center of the defect, and aim to provide a scaffold of interconnected pores with pore dimensions ranging from 200 μm [18, 19] to 2 mm [20], to facilitate tissue and vessel ingrowths. Among them, porous bioceramics made of calcium orthophosphates appear to be very prominent due to both the excellent biocompatibility and bonding ability to living bone in the body. This is directly related to the fact that the inorganic material of mammalian calcified tissues, *i.e.* of bone and teeth, consists of calcium orthophosphates [21-23]. Due to this reason, other artificial materials are normally encapsulated by fibrous tissue, when implanted in body defects, while calcium orthophosphates are not [24]. Several types of calcium orthophosphate-based bioceramics with different chemical composition are already on the market [9, 25]. Unfortunately, as for any ceramic material, calcium orthophosphate bioceramics by itself lack the mechanical and elastic properties of the calcified tissues; namely, scaffolds made of calcium orthophosphates only suffer from a low elasticity, a high brittleness, a poor tensile strength, a low mechanical reliability and fracture toughness, which leads to the concerns about their mechanical performance after implantation [26-28]. Besides, in many cases, it is difficult to form calcium orthophosphate bioceramics into the desired shapes.

Table 1. The biochemical composition* of bone [32].

Inorganic phases	wt. %	Bioorganic phases	wt. %
calcium orthophosphates (biological apatite)	~ 60	collagen type I	~ 20
water	~ 9	non-collagenous proteins: osteocalcin, osteonectin, osteopontin, thrombospondin, morphogenetic proteins, sialoprotein, serum proteins	~ 3
carbonates	~ 4	other traces: polysaccharides, lipids, cytokines	balance
citrates	~ 0.9	primary bone cells: osteoblasts, osteocytes, osteoclasts	balance
sodium	~ 0.7		
magnesium	~ 0.5		
other traces: Cl^- , F^- , K^+ , Sr^{2+} , Pb^{2+} , Zn^{2+} , Cu^{2+} , Fe^{2+}	balance		

* The composition is varied from species to species and from bone to bone.

The superior strength and partial elasticity of biological calcified tissues (*e.g.*, bones) are due to the presence of bioorganic polymers (mainly, collagen type I fibers¹) rather than to a natural ceramic (mainly, a poorly crystalline ion-substituted calcium deficient hydroxyapatite, often referred to as “biological apatite”) phase [30, 31]. The elastic collagen fibers are aligned in bone along the main stress directions. The biochemical composition of bone is given in Table 1 [32]. A decalcified bone becomes very flexible being easily twisted, whereas a bone without collagen is very brittle; thus, the inorganic nanocrystals of biological apatite provide

¹ One molecule of collagen type I is a triple helix with 338 repetitions of amino acid residues and is about 300 nm in length [29]. Additionally, bone contains small quantities of other bioorganic materials, such as proteins, polysaccharides and lipids, as well as bone contains cells and blood vessels.

with the hardness and stiffness, while the bioorganic fibers are responsible for the elasticity and toughness [22, 33]. In bones, both types of materials integrate each other into a nanometric scale in such a way that the crystallite size, fibers orientation, short-range order between the components, *etc.* determine its nanostructure and therefore the function and mechanical properties of the entire composite [29, 34-38]. From the mechanical point of view, bone is a tough material at low strain rates but fractures more like a brittle material at high strain rates; generally, it is rather weak in tension and shear, particularly along the longitudinal plane. Besides, bone is an anisotropic material because its properties are directionally dependent [21, 22, 28].

It remains a great challenge to design the ideal bone graft that emulates nature's own structures or functions. Certainly, the successful design requires an appreciation of the structure of bone. According to expectations, the ideal bone graft should be benign, available in a variety of forms and sizes, all with sufficient mechanical properties for use in load-bearing sites, form a chemical bond at the bone/implant interface, as well as be osteogenic, osteoinductive, osteoconductive, biocompatible, completely biodegradable at the expense of bone growth and moldable to fill and restore bone defects [26, 36, 39]. Further, it should resemble the chemical composition of bones (thus, the presence of calcium orthophosphates is mandatory), exhibit contiguous porosity to encourage invasion by the live host tissue, as well as possess both viscoelastic and semi-brittle behavior, as bones do [40-43]. Moreover, the degradation kinetics of the ideal implant should be adjusted to the healing rate of the human tissue with absence of any chemical or biological irritation and/or toxicity caused by substances, which are released due to corrosion or degradation. Ideally, the combined mechanical strength of the implant and the ingrowing bone should remain constant throughout the regenerative process. Furthermore, the substitution implant material should not disturb significantly the stress environment of the surrounding living tissue [44]. Finally, there is an opinion, that in the case of a serious trauma, bone should fracture rather than the implant [26]. A good sterilizability, storability and processability, as well as a relatively low cost are also of a great importance to permit a clinical application. Unfortunately, no artificial biomaterial is yet available, which embodies all these requirements and unlikely it will appear in the nearest future. To date, most of the available biomaterials appear to be either predominantly osteogenic or osteoinductive or else purely osteoconductive [2].

Careful consideration of the bone type and mechanical properties are needed to design bone substitutes. Indeed, in high load-bearing bones such as the femur, the stiffness of the implant needs to be adequate, not too stiff to result in strain shielding, but rigid enough to present stability. However, in relatively low load-bearing applications such as cranial bone repairs, it is more important to have stability and the correct three-dimensional shapes for aesthetic reasons. One of the most promising alternatives is to apply materials with similar composition and nanostructure to that of bone tissue [36]. Mimicking the structure of calcified tissues and addressing the limitations of the individual materials, development of organic-inorganic hybrid biomaterials provides excellent possibilities for improving the conventional bone implants. In this sense, suitable biocomposites of tailored physical, biological and mechanical properties with the predictable degradation behavior can be prepared combining biologically relevant calcium orthophosphates with bioresorbable polymers [45, 46]. As a rule, the general behavior of these bioorganic/calcium orthophosphate composites is dependent on nature, structure and relative contents of the constitutive components, although other parameters such as the preparation conditions also determine the

properties of the final materials. Currently, biocomposites with calcium orthophosphates incorporated as either a filler or a coating (or both) either into or onto a biodegradable polymer matrix, in the form of particles or fibers, are increasingly considered for using as bone tissue engineering scaffolds due to their improved physical, biologic and mechanical properties [47-53]. In addition, such biocomposites could fulfill general requirements to the next generation of biomaterials, those should combine the bioactive and bioresorbable properties to activate *in vivo* mechanisms of tissue regeneration, stimulating the body to heal itself and leading to replacement of the implants by the regenerating tissue [46, 54, 55]. Thus, through the successful combinations of ductile polymer matrixes with hard and bioactive particulate bioceramic fillers, optimal materials can be designed and, ideally, this approach could lead to a superior construction to be used as either implants or posterior dental restorative material [56].

A lint-reinforced plaster was the first composite used in clinical orthopedics as an external immobilizer (bandage) in the treatment of bone fracture by Mathijsen in 1852 [57], followed by Dreesman in 1892 [58]. A great progress in the clinical application of various types of composite materials has been achieved since then. Based on the past experience and newly gained knowledge, various composite materials with tailored mechanical and biological performance can be manufactured and used to meet various clinical requirements [59]. However, this book presents only a brief history and advances in the field of calcium orthophosphate-based biocomposites and hybrid biomaterials suitable for biomedical application. The majority of the reviewed literature is restricted to the recent publications; a limited number of papers published in the XX-th century have been cited. Various aspects of the material constituents, fabrication technologies, structural and bioactive properties, phase interaction have been considered and discussed in details. Finally, several critical issues and scientific challenges that are needed for further advancement are outlined.

GENERAL INFORMATION ON COMPOSITES AND BIOCOMPOSITES

According to Wikipedia, the free encyclopedia, “*composite materials* (or *composites* for short) are engineered materials made from two or more constituent materials with significantly different physical or chemical properties and which remain separate and distinct on a macroscopic level within the finished structure” [60]. Thus, composites are always heterogeneous. Following the point of view of some predecessors, we also consider that “for the purpose of this book, composites are defined as those having a distinct phase distributed through their bulk, as opposed to modular or coated components” [61, page 1329]. For this reason, with a few important exceptions, the structures obtained by soaking of various materials in supersaturated solutions containing ions of calcium and orthophosphate (*e.g.*, Refs. [62-67]), those obtained by coating of various materials by calcium orthophosphates (*e.g.*, Refs. [68-73]), as well as calcium orthophosphates coated by other compounds [74] have not been considered; however, composite coatings have been considered. Occasionally, porous calcium orthophosphate scaffolds filled by cells inside the pores [75, 76], as well as calcium orthophosphates impregnated by biologically active substances [77] are also defined as composites; nevertheless, such structures have not been considered in this book either.

In any composite, there are two major categories of constituent materials: a matrix (or a continuous phase) and (a) dispersed phase(s). To create a composite, at least one portion of each type is required. General information on the major fabrication and processing techniques might be found elsewhere [61]. The continuous phase is responsible for filling the volume, as well as it surrounds and supports the dispersed material(s) by maintaining their relative positions. The dispersed phase(s) is(are) usually responsible for enhancing one or more properties of the matrix. Most of the composites target an enhancement of mechanical properties of the matrix, such as stiffness and strength; however, other properties, such as erosion stability, transport properties (electrical or thermal), radiopacity, density or biocompatibility might also be of a great interest. This synergism produces the properties, which are unavailable from the individual constituent materials [78]. What's more, by controlling the volume fractions and local and global arrangement of the dispersed phase, the properties and design of composites can be varied and tailored to suit the necessary conditions. For example, in the case of ceramics, the dispersed phase serves to impede crack growth. In this case, it acts as reinforcement. A number of methods, including deflecting crack tips, forming bridges across crack faces, absorbing energy during pullout and causing a redistribution of stresses in regions adjacent to crack tips, can be used to accomplish this [79]. Other factors to be considered in composites are the volume fraction of (a) dispersed phase(s), its(their) orientation and homogeneity of the overall composite. For example, higher volume fractions of reinforcement phases tend to improve the mechanical properties of the composites, while continuous and aligned fibers best prevent crack propagation with the added property of anisotropic behavior. Furthermore, the uniform distribution of the dispersed phase is also desirable, as it imparts consistent properties to the composite [60, 78].

In general, composites might be simple, complex, graded and hierarchical. The term “a simple composite” is referred to the composites those result from the homogeneous dispersion of one dispersed phase throughout a matrix. The term “a complex composite” is referred to the composites those result from the homogeneous dispersion of several dispersed phases throughout one matrix. The term “a graded composite” is referred to the composites those result from the intentionally structurally inhomogeneous dispersion of one or several dispersed phases throughout one matrix. The term “a hierarchical composite” is referred to the cases, when fine entities of either a simple or a complex composite is somehow aggregated to form coarser ones (*e.g.*, granules or particles) which afterwards are dispersed inside another matrix to produce the second hierarchical scale of the composite structure. Another classification type of the available composites is based on either the matrix materials (metals, ceramics and polymers) or the reinforcement dimensions/shapes (particulates, whiskers/short fibers and continuous fibers) [59].

In most cases, three interdependent factors must be considered in designing of any composite: (i) selection of the suitable matrix and dispersed materials, (ii) choice of appropriate fabrication and processing methods, (iii) internal and external design of the device itself [61]. Besides, any composite must be formed to shape. To do this, the matrix material can be added before or after the dispersed material has been placed into a mold cavity or onto the mold surface. The matrix material experiences a melding event, that, depending upon the nature of the matrix material, can occur in various ways such as chemical polymerization, setting, curing or solidification from a melted state. Due to a general inhomogeneity, the physical properties of many composite materials are not isotropic but

rather orthotropic (*i.e.*, there are different properties or strengths in different orthogonal directions) [60, 78].

Biocomposites are defined as the composites able to interact well with the human body *in vivo* and, ideally, contain one or more component that stimulates the healing process and uptake of the implant. Thus, for biocomposites the biological compatibility appears to be more important than any other type of compatibility [59]. The most common properties from the bioorganic and inorganic domains to be combined in biocomposites have been summarized in Table 2 [36]. In 1990, Williams summarized the major types of biocomposites that were used in orthopedic applications that time [80]. In 2003, Wang published an excellent update [81]. For general advantages of the modern calcium orthophosphate-based biocomposites over calcium orthophosphate bioceramics and bioresorbable polymers individually, the interested readers are advised to get through “Composite materials strategy” chapter of Ref. [46].

Table 2. General respective properties from the bioorganic and inorganic domains, to be combined in various composites and hybrid materials [36].

inorganic	bioorganic
Hardness, brittleness	Elasticity, plasticity
High density	Low density
Thermal stability	Permeability
Hydrophilicity	Hydrophobicity
High refractive index	Selective complexation
Mixed valence slate (red-ox)	Chemical reactivity
Strength	Bioactivity

THE MAJOR CONSTITUENT MATERIALS OF BIOCOMPOSITES FOR BIOMEDICAL APPLICATIONS

3.1. Calcium Orthophosphates

The main driving force behind the use of calcium orthophosphates as bone substitute materials is their chemical similarity to the mineral component of mammalian bones and teeth [21-23]. As a result, in addition to being non-toxic, they are biocompatible, not recognized as foreign materials in the body and, most importantly, both exhibit bioactive behavior and integrate into living tissue by the same processes active in remodeling healthy bone. This leads to an intimate physicochemical bond between the implants and bone, termed osteointegration [81]. More to the point, calcium orthophosphates are also known to support osteoblast adhesion and proliferation [82, 83]. Even so, the major limitations to use calcium orthophosphates as load-bearing biomaterials are their mechanical properties; namely, they are brittle with poor fatigue resistance [26-28]. The poor mechanical behavior is even more evident for highly porous ceramics and scaffolds because porosity greater than 100 μm is considered as the requirement for proper vascularization and bone cell colonization [84-86].

That is why, in biomedical applications calcium orthophosphates are used primarily as fillers and coatings [23].

The complete list of known calcium orthophosphates, including their standard abbreviations and the major properties, is given in Table 3, while the detailed information on calcium orthophosphates, their synthesis, structure, chemistry, other properties and biomedical application has been comprehensively reviewed recently [23], where the interested readers are referred to. Even more thorough information might be found in various books and monographs [87-93].

Table 3. Existing calcium orthophosphates and their major properties [23].

Ca/P ionic ratio	Compound	Chemical formula	Solubility at 25 °C, – log(K_s)	Solubility at 25 °C, g/L	pH stability range in aqueous solutions at 25°C
0.5	Monocalcium phosphate monohydrate (MCPM)	$\text{Ca}(\text{H}_2\text{PO}_4)_2 \cdot \text{H}_2\text{O}$	1.14	~ 18	0.0–2.0
0.5	Monocalcium phosphate anhydrous (MCPA)	$\text{Ca}(\text{H}_2\text{PO}_4)_2$	1.14	~ 17	[c]
1.0	Dicalcium phosphate dihydrate (DCPD), mineral brushite	$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$	6.59	~ 0.088	2.0–6.0
1.0	Dicalcium phosphate anhydrous (DCPA), mineral monetite	CaHPO_4	6.90	~ 0.048	[c]
1.33	Octacalcium phosphate (OCP)	$\text{Ca}_8(\text{HPO}_4)_2(\text{PO}_4)_4 \cdot 5\text{H}_2\text{O}$	96.6	~ 0.0081	5.5–7.0
1.5	α -Tricalcium phosphate (α -TCP)	$\alpha\text{-Ca}_3(\text{PO}_4)_2$	25.5	~ 0.0025	[a]
1.5	β -Tricalcium phosphate (β -TCP)	$\beta\text{-Ca}_3(\text{PO}_4)_2$	28.9	~ 0.0005	[a]
1.2–2.2	Amorphous calcium phosphate (ACP)	$\text{Ca}_x\text{H}_y(\text{PO}_4)_z \cdot n\text{H}_2\text{O}$, $n = 3\text{--}4.5$; 15–20% H_2O	^[b]	^[b]	~5–12 ^[d]
1.5–1.67	Calcium-deficient hydroxyapatite (CDHA) ^[e]	$\text{Ca}_{10-x}(\text{HPO}_4)_x(\text{PO}_4)_{6-x}(\text{OH})_{2-x}$ ^[f] ($0 < x < 1$)	~85.1	~ 0.0094	6.5–9.5
1.67	Hydroxyapatite (HA)	$\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$	116.8	~ 0.0003	9.5–12
1.67	Fluorapatite (FA)	$\text{Ca}_{10}(\text{PO}_4)_6\text{F}_2$	120.0	~ 0.0002	7–12
2.0	Tetracalcium phosphate (TTCP), mineral hilgenstockite	$\text{Ca}_4(\text{PO}_4)_2\text{O}$	38–44	~ 0.0007	[a]

^[a] These compounds cannot be precipitated from aqueous solutions.

^[b] Cannot be measured precisely. However, the following values were found: 25.7±0.1 (pH=7.40), 29.9±0.1 (pH=6.00), 32.7±0.1 (pH=5.28).

^[c] Stable at temperatures above 100°C.

^[d] Always metastable.

^[e] Occasionally, CDHA is named as precipitated HA.

^[f] In the case $x = 1$ (the boundary condition with Ca/P = 1.5), the chemical formula of CDHA looks as follows: $\text{Ca}_9(\text{HPO}_4)(\text{PO}_4)_5(\text{OH})$.

3.2. Polymers

Polymers are a class of materials consisting of large molecules, often containing many thousands of small units, or monomers, joined together chemically to form one giant chain, thus creating very ductile materials. In this respect, polymers are comparable with major functional components of the biological environment: lipids, proteins and polysaccharides.

They differ from each other in chemical composition, molecular weight, polydispersity, crystallinity, hydrophobicity, solubility and thermal transitions. Besides, their properties can be fine-tuned over a wide range by varying the type of polymer, chain length, as well as by copolymerization or blending of two or more polymers [94, 95]. Opposite to ceramics, polymers exhibit substantial viscoelastic properties and easily can be fabricated into complex structures, such as sponge-like sheets, gels or complex structures with intricate porous networks and channels [96]. Being X-ray transparent and non-magnetic polymeric materials are fully compatible with the modern diagnostic methods such as computed tomography and magnetic resonance imaging. Unfortunately, most of them are unable to meet the strict demands of the *in vivo* physiological environment. Namely, the main requirements to polymers suitable for biomedical applications are that they must be biocompatible, not eliciting an excessive or chronic inflammatory response upon implantation and, for those that degrade, that they breakdown into non-toxic products only. Unfortunately, polymers, for the most part, lack rigidity, ductility and ultimate mechanical properties required in load bearing applications. Moreover, the sterilization processes (autoclave, ethylene oxide and ^{60}Co irradiation) may affect the polymer properties [97].

There is a variety of biocompatible polymers suitable for biomedical applications. For example, polyacrylates, poly(acrylonitrile-co-vinylchloride) and polylysine have been investigated for cell encapsulation and immunoisolation [98,99]. Polyorthoesters and PCL have been investigated as drug delivery devices, the latter for long-term sustained release because of their slow degradation rates [100]. PCL is a hydrolytic polyester having appropriate resorption period and releases nontoxic byproducts upon degradation [101]. Other polyesters and PTFE are used for vascular tissue replacement. Polyurethanes are in use as coatings for pacemaker lead insulation and have been investigated for reconstruction of the meniscus [102, 103]. Polymers considered for orthopedic purposes include polyanhydrides, which have also been investigated as delivery devices (due to their rapid and well-defined surface erosion), for bone augmentation or replacement since they can be photopolymerized *in situ* [100, 104, 105]. To overcome their poor mechanical properties, they have been copolymerized with imides or formulated to be crosslinkable *in situ* [105]. Other polymers, such as polyphosphazenes, can have their properties (*e.g.*, degradation rate) easily modified by varying the nature of their side groups and have been shown to support osteoblast adhesion, which makes them candidate materials for skeletal tissue regeneration [105]. PPF has emerged as a good bone replacement material, exhibiting good mechanical properties (comparable to trabecular bone), possessing the capability to crosslink *in vivo* through the C=C bond and being hydrolytically degradable. It has also been examined as a material for drug delivery devices [100, 104-107]. Polycarbonates have been suggested as suitable materials to make scaffolds for bone replacement and have been modified with tyrosine-derived amino acids to render them biodegradable [100]. Polydioxanone has been also tested for biomedical applications [108]. PMMA is widely used in orthopedics, as a bone cement for implant fixation, as well as to repair certain fractures and bone defects, for example, osteoporotic vertebral bodies [109]. However, PMMA sets by a polymerization of toxic monomers, which also evolves significant amounts of heat that damages tissues. Moreover, it is neither degradable nor bioactive, does not bond chemically to bones and might generate particulate debris leading to an inflammatory foreign body response [104, 110]. A number of other non-degradable polymers applied in orthopedic surgery include PE in its different modifications such as low density PE, HDPE and UHMWPE (used as the articular surface of

total hip replacement implants [111, 112]), polyethylene terephthalate, polypropylene and PTFE which are applied to repair knee ligaments [113]. Polyactive™, a block copolymer of PEG and PBT, was also considered for biomedical application [114-118]. Cellulose [119] and its esters [120] are also popular. Finally yet importantly, polyethylene oxide, PHB and blends thereof have also been tested for biomedical applications [46].

Table 4. Major properties of several FDA approved biodegradable polymers [121].

Polymer	Thermal properties*, °C	Tensile modulus, GPa	Degradation time, months
polyglycolic acid (PGA)	$t_g = 35-40$ $t_m = 225-230$	7.06	6-12 (strength loss within 3 weeks)
L-polylactic acid (LPLA)	$t_g = 60-65$ $t_m = 173-178$	2.7	>24
D,L-polylactic acid (DLPLA)	$t_g = 55-60$ amorphous	1.9	12-16
85/15 D,L-polylactic-co-glycolic acid (85/15 DLPLGA)	$t_g = 50-55$ amorphous	2.0	5-6
75/25 D,L-polylactic-co-glycolic acid (75/25 DLPLGA)	$t_g = 50-55$ amorphous	2.0	4-5
65/35 D,L-polylactic-co-glycolic acid (65/35 DLPLGA)	$t_g = 45-50$ Amorphous	2.0	3-4
50/50 D,L-polylactic-co-glycolic acid (50/50 DLPLGA)	$t_g = 45-50$ amorphous	2.0	1-2
poly(ϵ -caprolactone) (PCL)	$t_g = (-60)-(-65)$ $t_m = 58-63$	0.4	>24

* t_g – glass transition temperature; t_m – melting point.

Nonetheless, the most popular synthetic polymers used in medicine are the linear aliphatic poly(α -hydroxyesters) such as PLA, PGA and their copolymers – PLGA (Table 4). These materials have been extensively studied; they appear to be the only synthetic and biodegradable polymers with an extensive FDA approval history [46, 105, 121-125]. They are biocompatible, mostly non-inflammatory, as well as degrade *in vivo* through hydrolysis and possible enzymatic action into products that are removed from the body by regular metabolic pathways [45, 100, 105, 125-130]. Besides, they might be used for drug-delivery purposes [131]. Poly(α -hydroxyesters) have been investigated as scaffolds for replacement and regeneration of a variety of tissues, cell carriers, controlled delivery devices for drugs or proteins (*e.g.*, growth factors), membranes or films, screws, pins and plates for orthopedic applications [100, 105, 122, 103, 125, 132-134]. Additionally, the degradation rate of PLGA can be adjusted by varying the amounts of the two component monomers (Table 4), which in orthopedic applications can be exploited to create materials that degrade in concert with bone ingrowth [129, 135]. Furthermore, PLGA is known to support osteoblast migration and proliferation [55, 105, 126, 136], which is a necessity for bone tissue regeneration. Unfortunately, such polymers on their own, though they reduce the effect of stress-shielding, are too weak to be used in load bearing situations and are only recommended in certain clinical indications, such as ankle and elbow fractures [125, 130]. In addition, they exhibit

bulk degradation, leading to both a loss in mechanical properties and lowering of the local solution pH that accelerates further degradation in an autocatalytic manner. As the body is unable to cope with the vast amounts of implant degradation products, this might lead to an inflammatory foreign body response [105, 125, 132]. Finally, poly(α -hydroxyesters) do not possess the bioactive and osteoconductive properties of calcium orthophosphates [122, 137].

Several classifications of the biomedically relevant polymers are possible. For example, some authors distinguish between synthetic polymers like PLA and PGA or their copolymers with PCL, and polymers of biological origin like polysaccharides (starch, alginate, chitin/chitosan² [138-140], gelatin, cellulose, hyaluronic acid derivatives), proteins (soy, collagen, fibrin [9], silk) and a variety of biofibers, such as lignocellulosic natural fibers [8, 141, 142]. Other authors differentiate between resorbable or biodegradable (*e.g.*, poly(α -hydroxyesters), polysaccharides and proteins) and non-resorbable (*e.g.*, PE, PMMA and cellulose) polymers [56, 142]. As synthetic polymers can be produced under the controlled conditions, they in general exhibit predictable and reproducible mechanical and physical properties such as tensile strength, elastic modulus and degradation rate. Control of impurities is a further advantage of synthetic polymers. The list of synthetic biodegradable polymers used for biomedical application as scaffold materials is available as Table 1 in Ref. [142], while further details on polymers suitable for biomedical applications are available in literature [97, 134, 143-152] where the interested readers are referred. Good reviews on the synthesis of different biodegradable polymers [153], as well as on the experimental trends in polymer nanocomposites [154] are available elsewhere.

3.3. Inorganic Materials and Compounds (Metals, Ceramics, Glass, Oxides, Carbon, Etc.)

Titanium (Ti) is one of the best biocompatible metals and used most widely as implant [13,155]. Besides, there are other metallic implants made of pure Zr, Hf, V, Nb, Ta, Re [155], Ni, Fe, Cu [156, 157, 158], Ag, stainless steels and various alloys [158] suitable for biomedical application. Recent studies revealed even a greater biomedical potential of porous metals [159-161]. The metallic implants provide the necessary strength and toughness that are required in load-bearing parts of the body and, due to these advantages, metals will continue to play an important role as orthopedic biomaterials in the future, even though there are concerns with regard to the release of certain ions from and corrosion products of metallic implants. Of course, neither metals nor alloys are biomimetic³ in terms of chemical composition because there are no elemental metals in the human body. In addition, even biocompatible metals are bioinert: while not rejected by the human body, any metallic implants cannot actively interact with the surrounding tissues. Nevertheless, in some cases (especially when they are coated by calcium orthophosphates; however, that is another story) the metallic implants can show a reasonable biocompatibility [163]. Only permanent implants are made of metals and alloys, in which degradation or corrosion is not desirable. However,

² Chitosan is a biodegradable and semicrystalline polysaccharide obtained from N-deacetylation of chitin, which is harvested from the exoskeleton of marine crustaceans.

³ The term biomimetic can be defined as a processing technique that either mimics or inspires the biological mechanism, in part or whole [162].

during recent years a number of magnesium alloys have been proposed which are aimed to degrade in the body in order to make room for the ingrowing bone [161, 164].

Special types of glasses and glass ceramics are also suitable materials for biomedical applications [165-167] and a special $\text{Na}_2\text{O}-\text{CaO}-\text{SiO}_2-\text{P}_2\text{O}_5$ glass named Bioglass® [11, 24, 27, 28, 168, 169] is the most popular among them. They are produced via standard glass production techniques and require pure raw materials. Bioglass® is a biocompatible and osteoconductive biomaterial. It bonds to bone without an intervening fibrous connective tissue interface and, due to these properties, it has been widely used for filling bone defects [170]. The primary shortcoming of Bioglass® is mechanical weakness and low fracture toughness due to an amorphous two-dimensional glass network. The bending strength of most Bioglass® compositions is in the range of 40–60 MPa, which is not suitable for major load-bearing applications. Making porosity in Bioglass®-based scaffolds is beneficial for even better resorption and bioactivity [171].

By heat treatment, a suitable glass can be converted into glass-crystal composites containing crystalline phase(s) of controlled sizes and contents. The resultant glass ceramics can have superior mechanical properties to the parent glass as well as to sintered crystalline ceramics. The bioactive apatite-wollastonite (A-W) glass ceramics is made from the parent glass in the pseudoternary system $3\text{CaO} \cdot \text{P}_2\text{O}_5 - \text{CaO} \cdot \text{SiO}_2 - \text{MgO} \cdot \text{CaO} \cdot 2\text{SiO}_2$, which is produced by a conventional melt-quenching method. The bioactivity of A-W glass ceramics is much higher than that of sintered HA. It possesses excellent mechanical properties and has therefore been used clinically for iliac and vertebrae prostheses and as intervertebral spacers [13, 172, 173].

Metal oxide ceramics, such as alumina (Al_2O_3 , high purity, polycrystalline, fine grained) zirconia (ZrO_2) and some other oxides (*e.g.*, TiO_2), have been widely studied due to their bioinertness, excellent tribological properties, high wear resistance, fracture toughness and strength, as well as a relatively low friction [13, 174]. Unfortunately, due to transformation from the tetragonal to the monoclinic phase, a volume change occurs when pure zirconia is cooled down, which causes cracking of the zirconia ceramics. Therefore, additives such as calcia (CaO), magnesia (MgO) and yttria (Y_2O_3) must be mixed with zirconia to stabilize the material in either the tetragonal or the cubic phase. Such material is called PSZ [175-177]. However, the brittle nature of any ceramics has limited their scope of clinical applications and hence more research needs to be conducted to improve their properties.

CALCIUM ORTHOPHOSPHATE-BASED BIOCOMPOSITES AND HYBRID BIOMATERIALS

Generally, the use of calcium orthophosphate-based biocomposites and hybrid biomaterials for clinical applications has included several (partly overlapping) broad areas:

- biocomposites with polymers,
- cement-based biocomposites and concretes,
- nano-calcium orthophosphate-based biocomposites and nanocomposites,
- biocomposites with collagen,
- biocomposites with other bioorganic compounds and biological macromolecules,

- injectable bone substitutes (IBS),
- biocomposites with glasses, inorganic compounds and metals,
- functionally graded biocomposites,
- biosensors.

The details of each subject are given below.

4.1. Biocomposites with Polymers

Typically, the polymeric components of biocomposites and hybrid biomaterials comprise polymers that both have shown a good biocompatibility and are routinely used in surgical applications. In general, since polymers have a low modulus (2–7 GPa, as the maximum) as compared to that of bone (3–30 GPa), calcium orthophosphate bioceramics need to be loaded at a high weight % ratio. Besides, general knowledge on composite mechanics suggests that any high aspect ratio particles, such as whiskers or fibers, significantly improve the modulus at a lower loading [148]. Thus, some attempts have been already performed to prepare biocomposites containing whisker-like [178-181] or needle-like [182-184] calcium orthophosphates, as well as calcium orthophosphate fibers [45,185].

The history of implantable polymer-calcium orthophosphate biocomposites and hybrid biomaterials started in 1981⁴ from the pioneering study by Prof. William Bonfield and colleagues performed on HA/PE composites [187, 188]. That initial study introduced a bone-analogue concept, when proposed biocomposites comprised a polymer ductile matrix of PE and a ceramic stiff phase of HA, and was substantially extended and developed in further investigations by that research group [94, 189-206]. More recent studies included investigations on the influence of surface topography of HA/PE composites on cell proliferation and attachment [207-213]. The material is composed of a particular combination of HA particles at a volume loading of ~ 40% uniformly dispensed in a HDPE matrix. The idea was to mimic bone by using a polymeric matrix that can develop a considerable anisotropic character through adequate orientation techniques reinforced with a bone-like ceramics that assures both a mechanical reinforcement and a bioactive character of the composite. Following FDA approval in 1994, in 1995 this material has become commercially available under the trade-name HApEX™ (Smith and Nephew, Richards, USA), and to date remains the only clinically successful bioactive composite that appeared to be a major step in the implant field [28, 214]. The major production stages of HApEX™ include blending, compounding and centrifugal milling. A bulk material or device is then created from this powder by compression and injection molding [59]. Besides, HA/HDPE biocomposites might be prepared by a hot rolling technique that facilitated uniform dispersion and blending of the reinforcements in the matrix [215].

A mechanical interlock between the two phases of HApEX™ is formed by shrinkage of HDPE onto the HA particles during cooling [94, 216]. Both HA particle size and their distribution in the HDPE matrix were recognized as important parameters affecting the mechanical behavior of HApEX™ [198]. Namely, smaller HA particles were found to lead to stiffer composites due to general increasing of interfaces between the polymer and the

ceramics; furthermore, rigidity of HAPEX™ was found to be proportional to HA volume fraction [190]. In this formulation, HA could be replaced by other calcium orthophosphates [217].

Initial clinical applications of HAPEX™ came in orbital reconstruction [218] but since 1995, the main uses of this composite have been in the shafts of middle ear implants for the treatment of conductive hearing loss [219,220]. In both applications, HAPEX™ offers the advantage of *in situ* shaping, so a surgeon can make final alterations to optimize the fit of the prosthesis to the bone of a patient and subsequent activity requires only limited mechanical loading with virtually no risk of failure from insufficient tensile strength [94, 168]. As compared to cortical bones, HA/PE composites have a superior fracture toughness for HA concentrations below 40% and similar fracture toughness in the 45–50% range. Their Young's modulus is in the range of 1–8 GPa, which is quite close to that of bone. The examination of the fracture surfaces revealed that only mechanical bond occurs between HA and PE. Unfortunately, the HA/PE composites are not biodegradable, the available surface area of HA is low and the presence of bioinert PE decreases the ability to bond to bones. Furthermore, HAPEX™ has been designed with a maximized density to increase its strength but the resulting lack of porosity limits the ingrowth of osteoblasts when the implant is placed into the body [26,169]. Further details on HAPEX™ are available elsewhere [94]. Except of HAPEX™, other types of HA/PE biocomposites are also known [221–225].

Both linear and branched PE was used as a matrix and the biocomposites with the former were found to give a higher modulus [222]. The reinforcing mechanisms in calcium orthophosphate/polymer biocomposites have yet to be convincingly disclosed. Generally, if a poor filler choice is made, the polymeric matrix might be affected by the filler through reduction of molecular weight during composite processing, formation of an immobilized shell of polymer around the particles (transcrystallization, surface-induced crystallization or epitaxial growth) and changes in conformation of the polymer due to particle surfaces and inter-particle spacing [94]. On the other hand, the reinforcing effect of calcium orthophosphate particles might depend on the molding technique employed: a higher orientation of the polymeric matrix was found to result in a higher mechanical performance of the composite [226, 227].

Many other blends of calcium orthophosphates with various polymers are possible, including rather unusual formulations with dendrimers [228]. The list of the appropriate calcium orthophosphates is shown in Table 3 (except of MCPM and MCPA – both are too acidic and, therefore, are not biocompatible [23]), while many biomedically suitable polymers have been listed above. The combination of calcium orthophosphates and polymers into biocomposites has a twofold purpose. The desirable mechanical properties of polymers compensate for a poor mechanical behavior of calcium orthophosphate bioceramics, while in turn the desirable bioactive properties of calcium orthophosphates improve those of polymers, expanding the possible uses of each material within the body [127–129, 229–232]. Namely, polymers have been added to calcium orthophosphates in order to improve their mechanical strength [127, 229] and calcium orthophosphate fillers have been blended with polymers to improve their compressive strength and modulus, in addition to increasing their osteoconductive properties [48, 129, 137, 233–237]. Furthermore, biocompatibility of such biocomposites is enhanced because calcium orthophosphate fillers induce an increased initial

⁴ However, a more general topic “ceramic-plastic material as a bone substitute” is, at least, 18 years older [186].

flash spread of serum proteins compared to the more hydrophobic polymer surfaces [238]. What's more, experimental results of these biocomposites indicate favorable cell-material interactions with increased cell activities as compared to each polymer alone [231]. As a rule, with increasing of calcium orthophosphate content, both Young's modulus and bioactivity of the biocomposites increase, while the ductility decreases [26, 233]. Furthermore, such formulations can provide a sustained release of calcium and orthophosphate ions into the milieu, which is important for mineralized tissue regeneration [230]. Indeed, a combination of two different materials draws on the advantages of each one to create a superior biocomposite with respect to the materials on their own.

It is logical to assume that the proper biocomposite of a calcium orthophosphate (for instance, CDHA) with a bioorganic polymer (for instance, collagen) would yield the physical, chemical and mechanical properties similar to those of human bones. Different ways have been already realized to bring these two components together into composites, like mechanical blending, ball milling, dispersion of ceramic fillers into a polymer-solvent solution, a melt extrusion of a ceramic/polymer powder mixture, co-precipitation and electrochemical co-deposition [32, 59, 239-241]. Besides, there is *in situ* formation, which involves either synthesizing the reinforcement inside a preformed matrix material or synthesizing the matrix material around the reinforcement [59, 242]. For example, several papers have reported this method to produce various composites of apatites with carbon nanotubes [243-248]. Another example comprises using amino acid-capped gold nanoparticles as scaffolds to grow CDHA [249]. In certain cases, a mechano-chemical route [250], emulsions [251-254], freeze-drying [255] and freeze-thawing techniques [256], flame-sprayed technique [257] or gel-templated mineralization [258] might be applied to produce calcium orthophosphates-based biocomposites. Various fabrication procedures are available elsewhere [32, 59, 239], where the interested readers are referred.

The interfacial bonding between a calcium orthophosphate and a polymer is an important issue of any biocomposite. If adhesion between the phases is poor, the mechanical properties of a biocomposite suffer. To solve the problem, various approaches have been already introduced. For example, a diisocyanate coupling agent was used to bind PEG/PBT (PolyactiveTM) block copolymers to HA filler particles. Using surface-modified HA particles as a filler in a PEG/PBT matrix significantly improved the elastic modulus and strength of the polymer as compared to the polymers filled with ungrafted HA [235, 259]. Another group used processing conditions to achieve a better adhesion of the filler to the matrix. Ignjatovic *et al.* prepared PLLA/HA composites by pressing blends of varying PLLA and HA content at different temperatures and pressures [127, 128, 260]. They found that maximum compressive strength was achieved at ~ 15 wt.% of PLLA. By using blends with 20 wt.% of PLLA, the authors also established that increasing the pressing temperature and pressure improved the mechanical properties. The former was explained by decrease in viscosity of the PLLA associated with a temperature increase, hence leading to improved wettability of HA particles. The latter was explained by increased compaction and penetration of pores at higher pressure, in conjunction with a greater fluidity of the polymer at higher temperatures. The combination of high pressures and temperatures was found to decrease porosity and guarantee a close apposition of a polymer to the particles, thereby improving the compressive strength [229] and fracture energy [261] of the biocomposites. The PLLA/HA biocomposites scaffolds were found to improve cell survival over plain PLLA scaffolds [262].

It is also possible to introduce porosity into calcium orthophosphate-based biocomposites, which is advantageous for most applications as bone substitution material. The porosity facilitates the migration of osteoblasts from surrounding bones to the implant site [129, 263, 264]. Various material processing strategies to prepare composite scaffolds with interconnected porosity comprise thermally induced phase separation, solvent casting and particle leaching, solid freeform fabrication techniques, microsphere sintering and coating [142, 265-267]. A supercritical gas foaming technique might be used as well [239, 268, 269].

Apatite-Based Biocomposites

A biological apatite is known to be the major inorganic phase of mammalian calcified tissues [21, 22]. Consequently, CDHA, HA, carbonateapatite (both with and without dopants) and, occasionally, FA have been applied to prepare biocomposites with other compounds, usually with the aim to improve the bioactivity. For example, PS composed with HA can be used as a starting material for long-term implants [270-272]. Retrieved *in vivo*, HA/PS biocomposite coated samples from rabbit distal femurs demonstrated direct bone apposition to the coatings, as compared to the fibrous encapsulation that occurred when uncoated samples were used [270]. The resorption time of such biocomposites is a very important factor, which depends on polymer's microstructure and the presence of modifying phases [271].

Various apatite-containing biocomposites with PVA [256, 273-279], PVAP [282] and several other polymeric components [280, 281, 283-293] have been already developed. Namely, PVA/CDHA biocomposite blocks were prepared by precipitation of CDHA in aqueous solutions of PVA [256]. An artificial cornea consisted of a porous nano-HA/PVA hydrogel skirt and a transparent center of PVA hydrogel has been prepared as well. The results displayed a good biocompatibility and interlocking between artificial cornea and host tissues [277, 278]. PVAP has been chosen as a polymer matrix, because its phosphate groups can act as a coupling/anchoring agent, which has a higher affinity toward the HA surface [282]. Greish and Brown developed HA/Ca poly(vinyl phosphonate) biocomposites [284-286]. A template-driven nucleation and mineral growth process for the high-affinity integration of CDHA with PHEMA hydrogel scaffold has been developed as well [293].

PEEK [178,180,294-300] and HIPS [301] were applied to create biocomposites with HA having a potential for clinical use in load bearing applications. The study on reinforcing PEEK with thermally sprayed HA particles revealed that the mechanical properties increased monotonically with the reinforcement concentration, with a maximum value in the study of 40% volume fraction of HA particles [296-298]. The reported ranges of stiffness within 2.8–16.0 GPa and strength within 45.5–69 MPa exceeded the lower values for human bone (7–30 GPa and 50–150 MPa, respectively) [297]. Modeling of the mechanical behavior of HA/PEEK biocomposites is available elsewhere [299].

Biodegradable poly(α -hydroxyesters) are well established in clinical medicine. Currently, they provide with a good choice when a suitable polymeric filler material is sought. For example, HA/PLGA composites were developed which appeared to possess a cellular-compatibility suitable for bone tissue regeneration [302-309]. Zhang and Ma seeded highly porous PLLA foams with HA particles in order to improve the osteoconductivity of polymer scaffolds for bone tissue engineering [48, 234]. They pointed out that hydration of the foams prior to incubation in simulated body fluid increased the amount of carbonated CDHA material due to an increase of COOH and OH groups on the polymer surface, which

apparently acted as nucleation sites for apatite. The following values of Young's modulus, compressive, bending and tensile strengths for PLLA/HA composites have been achieved: 5–12 GPa, 78–137 MPa, 44–280 MPa and 10–30 MPa, respectively [310]. However, these data does not appear to be in a good agreement with HA/PLLA biocomposite unit cell model predictions [311].

On their own, PGA and PLA are known to degrade to acidic products (glycolic and lactic acids, respectively) that both catalyze polymer degradation and cause inflammatory reactions of the surrounding tissues [312]. Thus, in biocomposites of poly(α -hydroxyesters) with calcium orthophosphates, the presence of slightly basic compounds (HA, TTCP) to some extent neutralizes the acid molecules, provides with a weak pH-buffering effect at the polymer surface and, therefore, more or less compensates these drawbacks [137, 313–315]. However, additives of even more basic chemicals (*e.g.*, CaO, CaCO₃) might be necessary [142, 314, 316, 317]. Extensive cell culture experiments on pH-stabilized composites of PGA and carbonateapatite were reported, which afterwards were supported by extensive *in vitro* pH-studies [318]. A consequent development of this approach has led to designing of functionally graded composite skull implants consisting of polylactides, carbonateapatite and CaCO₃ [319, 320]. Besides the pH-buffering effect, inclusion of calcium orthophosphates was found to modify both surface and bulk properties of the biodegradable poly(α -hydroxyesters) by increasing the hydrophilicity and water absorption of the polymer matrix, thus altering the scaffold degradation kinetics. For example, polymer biocomposites filled with HA particles was found to hydrolyze homogeneously due to water penetrating into interfacial regions [321].

Biocomposites of poly(α -hydroxyesters) with calcium orthophosphates are mainly prepared by incorporating the inorganic phase into a polymeric solution, followed by drying under vacuum. The resulting solid composites might be shaped using different processing techniques. One can also prepare these biocomposites by mixing HA particles with L-lactide prior the polymerization [313] or by a combination of slip-casting technique and hot pressing [322]. A surfactant might be useful to keep the suspension homogeneity [323]. Besides, HA/PLA [252, 253] and HA/PLGA [254] microspheres might be prepared by a microemulsion technique. More complex carbonated-FA/PLA porous biocomposite scaffolds are also known [324]. An interesting list of references, assigned to the different ways of preparing HA/poly(α -hydroxyesters) biodegradable composites, might be found in publications by Durucan and Brown [49, 325, 326]. The authors prepared CDHA/PLA and CDHA/PLGA composites by solvent casting technique with a subsequent hydrolysis of α -TCP to CDHA in aqueous solutions. The presence of both polymers was found to inhibit α -TCP hydrolysis, if compared with that of single-phase α -TCP; what is more, the inhibiting effect of PLA exceeded that of PLGA [49, 325, 326]. The physical interactions between calcium orthophosphates and poly(α -hydroxyesters) might be easily seen in Fig. 1 [49]. Nevertheless, it should not be forgotten that typically non-melt based routes lead to development of composites with lower mechanical performance and many times require the use of toxic solvents and intensive hand labor [147].

The mechanical properties of poly(α -hydroxyesters) could be substantially improved by addition of calcium orthophosphates [327, 328]. Shikinami and Okuno developed CDHA/PLLA composites of very high mechanical properties [137]; mini-screws and mini-plates made of these composites have been manufactured and tested [321]. They have shown easy handling and shaping according to the implant site geometry, total resorbability, good

ability to bond directly to the bone tissue without interposed fibrous tissue, osteoconductivity, biocompatibility and high stiffness retainable for the period necessary to achieve bone union [321]. The initial bending strength of 280 MPa exceeded that of cortical bone (120–210 MPa), while the modulus was as high as 12 GPa [137]. The strength could be maintained above 200 MPa up to 25 weeks in phosphate-buffered saline solution. Such biocomposites were obtained from precipitation of a PLLA/dichloromethane solution, where small granules of uniformly distributed CDHA microparticles (average size of 3 μm) could be prepared [136]. Porous scaffolds of PDLLA and HA have been manufactured as well [269, 329, 330]. Upon implantation into rabbit femora, a newly formed bone was observed and biodegradation was significantly enhanced if compared to single-phase HA bioceramics. This might be due to a local release of lactic acid, which in turn dissolves HA. In other studies, PLA and PGA fibers were combined with porous HA scaffolds. Such reinforcement did not hinder bone ingrowth into the implants, which supported further development of such biocomposites as bone graft substitutes [47, 48, 310, 331, 332].

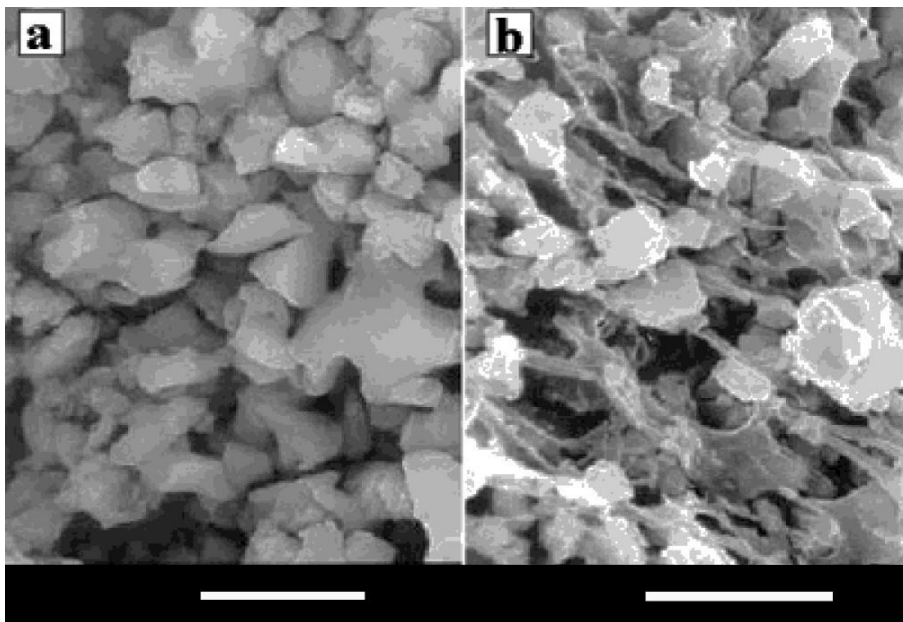


Figure 1. SEM micrographs of a) α -TCP compact; b) α -TCP-PLGA biocomposite (bars = 5 μm). Reprinted from Ref. [49] with permission.

Recently, blends (named as SEVA-C) of EVOH with starch filled with 10–30 wt.% HA have been fabricated to yield biocomposites with modulus up to ~ 7 GPa with a 30% HA loading [333–338]. The incorporation of bioactive fillers such as HA in SEVA-C aimed to assure the bioactive behavior of the composite and to provide the necessary stiffness within the typical range of human cortical bone properties. These biocomposites exhibited a strong *in vitro* bioactivity that was supported by the polymer's water-uptake capability [339]. However, the reinforcement of SEVA-C by HA particles was found to affect the rheological behavior of the blend. A degradation model of these biocomposites is available [340].

Higher homologues poly(3-hydroxybutyrate), 3-PHB, and poly(3-hydroxyvalerate), 3-PHV, show almost no biodegradation. Nevertheless, biocomposites of these polymers with

calcium orthophosphates showed a good biocompatibility both *in vitro* and *in vivo* [94,341-346]. Both bioactivity and mechanical properties of these biocomposites can be tailored by varying the volume percentage of calcium orthophosphates. Similarly, biocomposites of PHBV with both HA and amorphous carbonated apatite (almost ACP) appeared to have a promising potential for repair and replacement of damaged bones [347-350].

Along this line, PCL is used as a slowly biodegradable but well biocompatible polymer. PCL/HA composites have been already discussed as suitable materials for substitution, regeneration and repair of bone tissues [265, 351-358]. For example, biocomposites were obtained by infiltration of ϵ -caprolactone monomer into porous apatite blocks and *in situ* polymerization [354]. The composites were found to be biodegradable and might be applied as cancellous or trabecular bone replacement material or for cartilage regeneration. Both the mechanical performance and biocompatibility in osteoblast cell culture of PCL were shown to be strongly increased when HA was added [359]. Several preparation techniques of PCL/HA composites are known. For example, to make composite fibers of PCL/nano-HA, the desired amount of nano-HA powder was dispersed in a solvent using magnetic stirrer followed by ultrasonication for 30 min. Then, PCL was dissolved in this suspension, followed by the solvent evaporation [360]. The opposite preparation order is also possible: PCL was initially dissolved in chloroform at room temperature (7–10% weight/volume), then HA (~ 10 μ m particle size) was suspended in the solution, sonicated for 60 s, followed by the solvent evaporation [129] or salt-leaching [361]. The mechanical properties obtained by this technique were about one-third that of trabecular bone. In a comparative study, PCL and biological apatite were mixed in the ratio 19:1 in an extruder [362]. At the end of the preparation, the mixture was cooled in an atmosphere of nitrogen. The authors observed that the presence of biological apatite improved the modulus while concurrently increasing the hydrophilicity of the polymeric substrate. Besides, an increase in apatite concentration was found to increase both the modulus and yield stress of the composite, which indicated to good interfacial interactions between the biological apatite and PCL. It was also observed that the presence of biological apatite stimulated osteoblasts attachment to the biomaterial and cell proliferation [362]. In another study, a PCL/HA biocomposite was prepared by blending in melt form at 120°C until the torque reached equilibrium in the rheometer that was attached to the blender [363]. Then the sample was compression molded and cut into specimens of appropriate size for testing. It was observed that the composite containing 20 wt.% HA had the highest strength [363]. However, a direct grafting of PCL on the surface of HA particles seems to be the most interesting preparation technique [351]. HA porous scaffolds were coated by a PCL/HA composite coating [50]. In this system, PCL, as a coating component, was able to improve the brittleness and low strength of the HA scaffolds, while the particles in the coating were to improve the osteoconductivity and bioactivity of the coating layer. More complex PDLLA/PCL/HA biocomposites have been prepared as well [364]. Further details on both PCL/HA biocomposites and processing methodologies thereof might be found elsewhere [265].

The spread of attached human osteoblasts onto PLA and PCL films reinforced with CDHA and sintered HA was shown to be higher than for the polymers alone [153]. Moreover, biochemical assays relating cell activity to DNA content allowed concluding that cell activity was more intense for the composite films [153]. Kim *et al.* coated porous HA blocks with PCL from dichloromethane solution and performed drug release studies. The antibiotic

tetracycline hydrochloride was added into this layer, yielding a bioactive implant with drug release for longer than a week [50].

Yoon *et al.* investigated the highest mechanical and chemical stability of FA by preparing FA/collagen biocomposites and studied their effect in osteoblast-like cell culture [365]. The researchers found an increased cellular activity in FA composites compared to HA composites. This finding was confirmed in another study by means of variations in the fluoride content for FA-HA/PCL composites [366]. An interesting phenomenon of fractal growth of FA/gelatin composite crystals (Fig. 2) was achieved by diffusion of calcium- and orthophosphate+fluoride-solutions from the opposite sides into a tube filled with a gelatin gel [367-375]. The reasons of this phenomenon are not quite clear yet; besides, up to now nothing has yet been reported on a possible biomedical application of such very unusual structural composites.

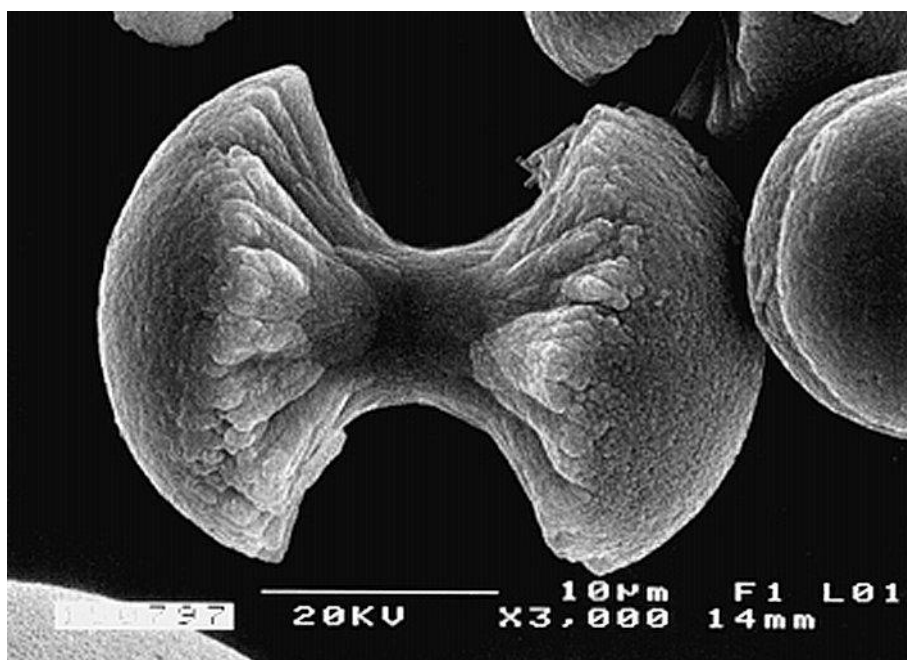


Figure 2. A biomimetically grown aggregate of FA that was crystallized in a gelatin matrix. Its shape can be explained and simulated by a fractal growth mechanism. Scale bar: 10 μm . Reprinted from Ref. [367] with permission.

TCP-Based Biocomposites

Both α -TCP and β -TCP have a higher solubility than HA (Table 3). Besides, they are faster resorbed *in vivo*.⁵ Therefore, these calcium orthophosphates were used instead of HA to prepare completely biodegradable biocomposites [377-395]. For example, a biodegradable and osteoconductive biocomposite made of β -TCP particles and gelatin was proposed [386]. This material was tested *in vivo* with good results. It was found to be biocompatible, osteoconductive and biodegradable with no need for a second surgical operation to remove

⁵ However, there are some reports about a lack of TCP biodegradation after implantation in calvarial defects [376].

the device after healing occurred. Herbal extracts might be added to this biocomposite [387]. Another research group prepared biocomposites of crosslinked gelatin with β -TCP; they found both a good biocompatibility and bone formation upon subcutaneous implantation in rats [388]. Yang *et al.* [393] extended this to porous (porosity about 75%) β -TCP/gelatin biocomposites those also contained BMP-4. Besides, cell-compatible and possessive some osteoinductive properties porous β -TCP/alginate-gelatin hybrid scaffolds were prepared and successfully tested *in vitro* [390]. More to the point, biocomposites of β -TCP with PLLA [383, 384] and PLGC [385] were prepared. Although β -TCP was able to counter the acidic degradation of the polyester to some extent, it did not prevent a pH drop down to ~ 6 . Nevertheless, implantation of this biocomposite in beagles' mandibular bones was successful [385].

Based on the self-reinforcement concept, biocomposites of TCP with polylactides were prepared and studied using conventional mechanical testing [396]. Bioresorbable scaffolds were fabricated from such biocomposites [397]. Chitosan was also used as the matrix for the incorporation of β -TCP by a solid/liquid phase separation of the polymer solution and subsequent sublimation of the solvent. Due to complexation of the functional groups of chitosan with calcium ions of β -TCP, these biocomposites had a better compressive modulus and strength [398]. PCL/ β -TCP biocomposites were developed as well [399-402] and their *in vitro* degradation behavior was systematically monitored by immersion in simulated body fluid at 37 °C [401]. To extend this topic further, the PCL/ β -TCP biocomposites might be loaded by drugs [402].

Cell culture tests on β -TCP/PLLA biocomposites were reported; the biocomposites showed no cytotoxicity and evidenced good cell attachment to its surface [377]. An *in vitro* study with primary rat calvarial osteoblasts showed an increased cellular activity in the BMP-loaded samples [393]. Other researchers investigated BMP-2-loaded porous β -TCP/gelatin biocomposites (porosity 95%, average pore size 180-200 μ m) [403] and confirmed the precious study. Biocomposites of β -TCP and glutaraldehyde cross-linked gelatin were manufactured and tested *in vitro* to measure the material cytotoxicity [389]. The experimental results revealed that the amount of glutaraldehyde cross-linking agent should be less than 8% to decrease the toxicity on the osteoblasts and to avoid inhibition of cellular growth caused by the release of residual or uncross-linked glutaraldehyde.

A long-term implantation study of PDLLA/ α -TCP composites in a loaded sheep implant model showed good results after 12 months but a strong osteolytic reaction after 24 months. This was ascribed to the almost complete dissolution of α -TCP to this time and an adverse reaction of the remaining PDLLA [404].

More complex calcium orthophosphate-based biocomposites are known as well. For example, there is a composite consisting of three interpenetrating networks: TCP, CDHA and PLGA [405]. Firstly, a porous TCP network was produced by coating a polyurethane foam by hydrolysable α -TCP slurry. Then, a CDHA network was derived from a calcium orthophosphate cement filled in the porous TCP network. Finally, the remaining open pore network in the CDHA/ α -TCP structures was infiltrated with PLGA. This biocomposite consists of three phases with different degradation behavior. It was postulated that bone would grow on the fastest degrading network of PLGA, while the remaining calcium orthophosphate phases would remain intact thus maintaining their geometry and load bearing capability [405].

Other Calcium Orthophosphate-Based Biocomposites

The number of research papers devoted to biocomposites based on other calcium orthophosphates is substantially lesser than those devoted to apatites and TCP. Biphasic calcium phosphate (BCP)⁶ appears to be most popular among the remaining calcium orthophosphates. Collagen coated BCP ceramics was studied and the biocompatibility towards osteoblasts was found to increase upon coating with collagen [406]. Another research group created porous PDLLA/BCP scaffolds and coated them with a hydrophilic PEG/vancomycin composite for both drug delivery purposes and surface modification [407]. More to the point, PLGA/BCP composites were fabricated [408, 409] and their cytotoxicity and fibroblast properties were found to be acceptable for natural bone tissue reparation, filling and augmentation [410, 411]. PCL/BCP biocomposites are known as well [412].

A choice of DCPD-based biocomposites of DCPD, albumin and duplex DNA was prepared by water/oil/water interfacial reaction method [251]. Core-shell type DCPD/chitosan biocomposite fibers were prepared by a wet spinning method in another study [413]. The energy-dispersive X-ray spectroscopy analysis indicated that Ca and P atoms were mainly distributed on the outer layer of the composite fibers; however, a little amount of P atoms remained inside the fibers. This indicated that the composite fibers formed a unique core-shell structure with shell of calcium orthophosphate and core of chitosan [413]. Although, this is not to the point, it is interesting to mention that some DCPD/polymer composites could be used as proton conductors in battery devices [414, 415]. Nothing has been reported on their biocompatibility but, perhaps, sometime the improved formulations will be used to fabricate biocompatible batteries for implantable electronic devices.

Various ACP-based biocomposites for dental applications were developed [416-419]. Besides, several ACP-based formulations were investigated as potential biocomposites for bone grafting [350, 420-422]. Namely, ACP/PPF biocomposites were prepared by *in situ* precipitation [421], while PHB/carbonated ACP and PHBHV/carbonated ACP biocomposites appeared to be well suited as slowly biodegradable bone substitution material [350]. Another example comprises hybrid nano-capsules of ~ 50-70 nm in diameter which were fabricated by ACP mineralization of shell cross-linked polymer micelles and nanocages [422]. These nano-capsules consisted of a continuous ultrathin inorganic surface layer that infiltrated the outer cross-linked polymeric domains. They might be used as structurally robust, pH-responsive biocompatible hybrid nanostructures for drug delivery, bioimaging and therapeutic applications [422].

4.2. Calcium Orthophosphate Cement-Based Biocomposites and Concretes

Inorganic self-setting calcium orthophosphate cements, which harden in the body, were introduced by LeGeros *et al.* [423] and Brown and Chow [424, 425] in the early 1980-s⁷. Since then, these cements have been broadly studied and many formulations have been

⁶ BCP is a solid composite of HA and β -TCP; however, similar composites of HA and α -TCP are possible as well [23].

⁷ There is an opinion [426] that the self-setting calcium orthophosphate cements for orthopedic and dental restorative applications have first been described in the early 1970-s by Driskell *et al.* in US Patent No. 3913229 [427].

proposed [428]. The cements set and harden due to various chemical interactions among calcium orthophosphates that finally lead to formation of a monolithic body consisting of either CHDA or DCPD with possible admixtures of other phases. Unfortunately, having the ceramic nature, calcium orthophosphate cements are brittle after hardening and the setting time is sometimes unsuitable for clinical procedures [428]. Therefore, various attempts have been performed to transform the cements into biocomposites *e.g.*, by adding hydroxylcarboxylic acids, to control the setting time [429], gelatin to improve both the mechanical properties and the setting time [392, 430-432] or osteocalcin/collagen to increase the bioactivity [433]. More to the point, various reinforcement additives of different shapes and nature are widely used to improve the mechanical properties of calcium orthophosphate cements [428]. Even carbon nanotubes were used for this purpose [434]! Although the biomaterials community does not use this term, a substantial amount of the reinforced cement formulations might be defined as calcium orthophosphate-based concretes⁸. The idea behind the concretes is simple: if a strong filler is present in the matrix, it might stop crack propagation.

Various apatite-containing biocomposite formulations based on PMMA [436-446] and PEMA [94,447,448] have been already developed. Such biocomposites might be prepared by dispersion of apatite powder into a PMMA viscous fluid [449] and used for drug-delivery purposes [450]. When the mechanical properties of the biocomposite concretes composed of PMMA matrix and HA particles of various sizes were tested, the tensile results showed that strength was independent on particle sizes. In addition, up to 40% by weight HA could be added without impairing the mechanical properties [439, 440]. After immersion into Ringer's solution, the tensile strength was not altered whereas the fatigue properties were significantly reduced. The biocompatibility of PMMA/HA biocomposites was tested *in vivo* and enhanced osteogenic properties of the implants compared to single-phase PMMA were observed [437, 441-444]. It was shown that not only the mechanical properties of PMMA were improved but the osteoblast response of PMMA was also enhanced with addition of HA [441]. Thereby, by adding of calcium orthophosphates, a non-biodegradable PMMA was made more bioactive and osteoconductive, yielding a well-processable biocomposite concrete. As a drawback, the PMMA/HA formulations possess a low flexural, compressive and tensile strength.

A biocomposite made from HA granules and bis-phenol- α -glycidylmethacrylate-based resin appeared to possess comparable mechanical and biological properties to typical PMMA cement, leading to potential uses for implant fixation [451]. To improve the mechanical properties of calcium orthophosphate cements and stabilize them at the implant site, various researchers have resorted to formulations that set *in situ*, primarily through crosslinking reactions of the polymeric matrix. For example, TTCP was reacted with PAA, forming a crosslinked CDHA/calcium polyacrylate biocomposite [452]. In aqueous solutions, TTCP hydrolyzes to CDHA [23] and the liberated calcium cations react with PAA, forming the crosslinked network [452]. Reed *et al.*, synthesized a dicarboxy polyphosphazene that can be crosslinked by calcium cations and cement-based (TTCP+DCPD) CDHA/polyphosphazene biocomposites with a compressive strength ~10 MPa and of ~65% porosity were prepared as

⁸ According to Wikipedia, the free encyclopedia: "Concrete is a construction material that consists of a cement (commonly Portland cement), aggregates (generally gravel and sand) and water. It solidifies and hardens after mixing and placement due to a chemical process known as hydration. The water reacts with the cement, which bonds the other components together, eventually creating a stone-like material" [435].

a result [453]. To mimic PMMA cements, PPF/ β -TCP biocomposites were prepared with addition of vinyl monomer to crosslink PPF. As a result, quick setting and degradable biocomposite cements with a low heat output and compressive strengths in the range of 1-12 MPa were prepared by varying the molecular weight of PPF, as well as the contents of the monomer, β -TCP, initiator and porogen (NaCl) [454, 455]. An acrylic cement with Sr-containing HA as a filler [110] and an injectable polydimethylsiloxane/HA cement [456] have been prepared as well.

In order to improve the mechanical properties of calcium orthophosphate cements, numerous researchers blended various polymers with the cements. For example, gelatin might be added to calcium orthophosphate cement formulations, primarily to stabilize the paste in aqueous solution before it develops adequate rigidity and, secondly, to improve the compressive strength [392, 430, 457]. Adding rod-like fillers to the cement formulations also caused an improvement in the mechanical properties [457]. For example, PAA and PVA were successfully used to improve the mechanical properties of a TTCP+DCPD cement but, unfortunately, with an inevitable and unacceptable reduction of both workability and setting time [458, 459]. Similar findings were reported in the presence of sodium alginate and sodium polyacrylate [460]. Other polymers, such as polyphosphazene might be used as well [461-463]. Other examples of polymer/calcium orthophosphate cement formulations might be found elsewhere [464, 465].

Porous calcium orthophosphate scaffolds with interconnected macropores (~ 1 mm), micropores (~ 5 μ m) and of high porosity ($\sim 80\%$) were prepared by coating polyurethane foams with a TTCP+DCPA cement, followed by firing at 1200 $^{\circ}$ C. In order to improve the mechanical properties of the scaffolds, the open micropores of the struts were then infiltrated by a PLGA solution to achieve an interpenetrating bioactive ceramic/biodegradable polymer composite structure. The PLGA filled struts were further coated with a 58S bioactive glass/PLGA composite coating. The obtained complex porous biocomposites could be used as tissue engineering scaffolds for low-load bearing applications [466]. A more complicated construction, in which the PLGA macroporous phase has been reinforced with a bioresorbable TTCP+DCPA cement, followed by surface coating of the entire construct by a non-stoichiometric CDHA layer, has been designed as well [467]. The latter approach has culminated in a unique, three-phase biocomposite that is simple to fabricate, osteoconductive and completely biodegradable.

A porosity level of 42–80% was introduced into calcium orthophosphate cement/chitosan biocomposites by addition of the water-soluble mannitol [468]. Chitosan significantly improved the mechanical strength of the entire biocomposite [469]. A similar approach was used by other researchers who studied the effect of the addition of PLGA microparticles [470-473] (which can also be loaded with drugs or growth factors [474-476]) to calcium orthophosphate cements. These biocomposites were implanted into cranial defects of rats and a content of ~ 30 wt.% of the microparticles was found to give the best results [470], while the addition of a growth factor to the biocomposites significantly increased bone contact at 2 weeks and enhanced new bone formation at 8 weeks [476]. The *in vivo* rabbit femur implant tests showed that PLGA/calcium orthophosphate cement formulations exhibited outstanding biocompatibility and bioactivity, as well as a better osteoconduction and degradability than pure calcium orthophosphate cements [471]. Further details on calcium orthophosphate cement-based biocomposites and concretes might be found in Ref. [428, chapter “Reinforced calcium orthophosphate cements”].

4.3. Nano-Calcium Orthophosphate-Based Biocomposites and Nano-Biocomposites

Nanophase materials are the materials that have grain sizes under ~ 100 nm. They have different mechanical and optical properties if compared to the large grained materials of the same chemical composition. Namely, nanophase materials have the unique surface properties, such as an increased number of atoms, grain boundaries and defects at the surface, huge surface area and altered electronic structure, if compared to the conventional micron-sized materials. For example, nano-HA (size ~ 67 nm) has a higher surface roughness of 17 nm if compared to 10 nm for the conventional submicron size HA (~ 180 nm), while the contact angles (a quantitative measure of the wetting of a solid by a liquid) are significantly lower for nano-HA (6.1) if compared to the conventional HA (11.51). Additionally, the diameter of individual pores in a nano-HA compact is five-times smaller (pore diameter ~ 6.6 Å) than that in the conventional grain-sized HA compacts (pore diameter within 19.8–31.0 Å) [477-479]. Besides, nano-HA promotes osteoblast cells adhesion, differentiation and proliferation, osteointegration and deposition of calcium containing minerals on its surface better than microcrystalline HA; thus enhancing formation of a new bone tissue within a short period [477-479]. More to the point, nano-HA was found to cause apoptosis of the leukemia P388 cells [480].

Composites of two or more materials, in which at least one of the materials is of a nanometer-scale, are defined as *nanocomposites* [32]. Natural bone mineral is a hierarchical nanocomposite of biological origin because it consists of nano-sized blade-like crystals of biological apatite grown in intimate contact with an organic matrix rich in collagen fibers and organized in a complicated hierarchical structure [21, 22, 38]. Given the fact that the major organic phase of bone is collagen, *i.e.* a natural polymer (Table 1), it is obvious that a composite of a nanophase calcium orthophosphate with a biodegradable polymer should be advantageous as bone substitution material. The inorganic nanophase would be responsible for the mechanical strength (hardness) and bioactivity, while the polymer phase would provide the elasticity. In addition, the solubility of calcium orthophosphates depends on their crystallite size (smaller crystals have a higher solubility) and on their carbonate content (higher carbonate content increases the solubility) [481]. To the author's best knowledge, among calcium orthophosphates listed in Table 3, before very recently only apatites (CDHA, HA and, perhaps, FA) have been available in the nanocrystalline state. However, very recently, nano-DCPA [482-484] and nano-MCPM [485] have been synthesized and applied to prepare nano-biocomposites with strong ionic release to combat tooth caries.

A number of investigations have been conducted recently to determine the mineralization, biocompatibility and mechanical properties of the nano-biocomposites based on various (bio)polymers and nano-HA.⁹ These studies covered nano-HA/PLA [269,486-493] and its copolymer with PGA [494-496], nano-HA/collagen [497-509], nano-HA/collagen/PLA [509-517], nano-HA/collagen/PVA [518], nano-HA/collagen/alginate [519, 520], nano-HA/gelatin [521-526], nano-HA/poly(hexamethylene adipamide) [527], nano-HA/PPF [528], nano-HA/polyamide [529-540], nano-HA/PVA [277, 278, 541-543], nano-HA/PVAP [282], nano-HA/poly(ethylene-co-acrylic) acid [544, 545], nano-HA/chitosan

⁹ Unfortunately, in the majority of the already published papers it often remained unclear whether “nano-HA” represented the stoichiometric nano-HA or a non-stoichiometric nano-CDHA.

[546-549], nano-HA/konjac glucomannan/chitosan [550], nano-HA/PHEMA/PCL [551], nano-HA/PCL [323,360,552,553], nano-HA/Ti [554, 555], PCL semi-interpenetrating nanocomposites [556] and many other biocompatible hybrid formulations [224, 258, 272, 348, 557-575]. Several nano-biocomposites were found to be applicable as carriers for growth factors delivery [34, 576, 577]. Besides, the data are available on the excellent biocompatibility of such nano-biocomposites [508]. The dispersion state of nanoparticles appears to be the critical parameter in controlling the mechanical properties of nano-biocomposites, as nanoparticles always tend to aggregate owing to their high surface energy [348].

Porous (porosity ~85%) biocomposites of nano-HA with collagen and PLA have been prepared by precipitation and freeze-drying; the nano-biocomposites did not show a pH drop upon *in vitro* degradation [510-512]. They were implanted in the radius of rabbits and showed a high biocompatibility and partial resorption after 12 weeks. Nano-HA/chitosan biocomposites with improved mechanical stability were prepared from HA/chitosan nanorods [578]. Nano-HA/PLLA biocomposites of high porosity (~90%) were prepared using thermally induced phase separation [579]. Besides, nano-HA was used to prepare biocomposites with PAA and the nanostructure of the resulting nanocrystals exhibited a core-shell configuration [580, 581].

Nano-HA crystals appeared to be suitable for intraosseous implantation and offered a potential to formulate enhanced biocomposites for clinical applications [582]. Thus, the biocompatibility of chitosan in osteoblast cell culture was significantly improved by addition of nano-HA [583]. Similar finding is valid for nano-HA/polyamide biocomposites [532]. Further details on nano-HA-based biocomposites might be found in an excellent review [32]. More to the point, a more general review on nanobiomaterial applications in orthopedics is also available [584], where the interested readers are referred.

4.4. Biocomposites with Collagen

The main constituent of the bioorganic matrix of bones is type I collagen¹⁰ (Table 1) with molecules about 300 nm in length. This protein is conducive to crystal formation in the associated inorganic matrix. It is easily degraded and resorbed by the body and allows good attachment to cells. Collagen alone is not effective as an osteoinductive material but it becomes osteoconductive in combination with calcium orthophosphates [586]. Both collagen type I and HA were found to enhance osteoblast differentiation [587] but combined together, they were shown to accelerate osteogenesis. However, this tendency is not so straightforward: the data are available that implanted HA/collagen biocomposites enhanced regeneration of calvaria bone defects in young rats but postponed the regeneration of calvaria bone in aged rats [588]. Finally, addition of calcium orthophosphates to collagen sheets was found to give a higher stability and an increased resistance to 3D swelling compared to the collagen reference [589]. Therefore, a bone analogue based on these two constituents should possess the remarkable properties. Furthermore, addition of bone marrow constituents gives osteogenic and osteoinductive properties to calcium orthophosphate/collagen biocomposites [1].

¹⁰ The structural and biochemical properties of collagens have been widely investigated and over 25 collagen subtypes have been identified [585].

The unique characteristics of bones are the spatial orientation between the calcium orthophosphate nanophase and collagen macromolecules at the nanolevel [35], where nanocrystals (about 50 nm length) of biological apatite are aligned parallel to the collagen fibrils [21, 22, 31, 38], which is believed to be the source of the mechanical strength of bones. The collagen molecules and the nanocrystals of biological apatite assembled into mineralized fibrils are approximately 6 nm in diameter and 300 nm long [31, 35, 38, 511, 590]. Although the complete mechanisms involved in the bone building strategy are still unclear, the strengthening effect of apatite nanocrystals in calcified tissues might be explained by the fact that the collagen matrix is a load transfer medium and thus transfers the load to the intrinsically rigid inorganic nanocrystals. Furthermore, nanocrystals of biological apatite located in between tangled fibrils cross-link the fibers either through a mechanical interlocking or by forming calcium ion bridges, thus increasing deformation resistance of the collagenous fiber network [591].

When calcium orthophosphates are combined with collagen in a laboratory, the biocomposites appear to be substantially different from natural bone tissue due to a lack of real interaction between the two components, *i.e.* interactions that are able to modify the intrinsic characteristics of the singular components themselves. The main characteristics of the route, by which the mineralized hard tissues are formed *in vivo*, is that the organic matrix is laid down first and the inorganic reinforcing phase grows within this organic matrix [21, 22, 31, 38]. Although to date, neither the elegance of the biomineral assembly mechanisms nor the intricate composite nano-architectures have been duplicated by non-biological methods, the best way to mimic bone is to copy the way it is formed, namely by nucleation and growth of CDHA nanocrystals from a supersaturated solution both onto and within the collagen fibrils [592-594]. Such syntheses were denoted as “biologically inspired” which means they reproduce an ordered pattern and an environment very similar to natural ones [595-597]. The biologically inspired biocomposites of collagen and calcium orthophosphates (mainly, apatites) for bone substitute have a long history [29, 365, 500, 598-616] and started from the pioneering study by Mittelmeier and Nizard [617], who mixed calcium orthophosphate granules with a collagen web. Such combinations were found to be bioactive, osteoconductive, osteoinductive [29, 586, 618-620] and, in general, artificial grafts manufactured from this type of the biocomposites are likely to behave similarly to bones and be of more use in surgery than those prepared from any other materials. Indeed, some data are available on the superiority of calcium orthophosphate/collagen biocomposite scaffolds over the artificial polymeric and calcium orthophosphate bioceramic scaffolds individually [621].

It has been found that calcium orthophosphates may be successfully precipitated onto a collagen substrate of whatever form or source [29, 36, 500, 622, 623]. However, adherence of calcium orthophosphate crystals to collagen did depend on how much the collagen had been denatured: the more fibrillar the collagen, the greater attachment. Clarke *et al.* first reported the production of a biocomposite produced by precipitation of DCPD onto a collagen matrix with the aid of phosphorylated amino acids commonly associated with fracture sites [603]. Apatite cements (DCPD+TTCP) have been mixed with a collagen suspension, hydrated and allowed to set. CDHA crystals were found to nucleate on the collagen fibril network, giving a material with the mechanical properties weaker than those reported for bone. More to the point, these biocomposites were without the nanostructure similar to that of bone [600, 624]. The oriented growth of OCP crystals on collagen was achieved by an experimental device in which Ca^{2+} and PO_4^{3-} ions diffused into a collagen disc from the opposite directions [623,

625, 626]. Unfortunately, these experiments were designed to simulate the mechanism of *in vivo* precipitation of biological apatite only; due to this reason, the mechanical properties of the biocomposites were not tested [627].

Conventionally, collagen/calcium orthophosphate biocomposites can be prepared by blending or mixing of collagen and calcium orthophosphates, as well as by biomimetic methods [29, 32, 34, 37, 497, 500, 511, 577, 590, 595-597, 600, 622, 628-634]. Besides, collagen might be incorporated into calcium orthophosphate cements [600, 624, 635]. Typically, the type I collagen sponge is presoaked in PO_4^{3-} -containing a highly basic aqueous solution and then is immersed into a Ca^{2+} -containing solution to allow mineral deposition. Also, collagen I fibers might be dissolved in acetic acid and then this solution is added to phosphoric acid, followed by the neutralization synthesis (performed at 25 °C and solution pH within 9 – 10) between an aqueous suspension of $\text{Ca}(\text{OH})_2$ and the H_3PO_4 /collagen solution [595, 596]. To ensure the quality of the final product, it is necessary to control the Ca/P ionic ratio in the reaction solution. One way to do this is to dissolve a commercial calcium orthophosphate in an acid; another one is to add Ca^{2+} and PO_4^{3-} ions in a certain ratio to the solution and after that induce the reaction [35]. Biomimetically, one can achieve an oriented growth of CDHA crystals onto dissolved collagen fibrils in aqueous solutions via a self-organization mechanism [629]. A number of authors produced calcium orthophosphate/collagen biocomposites by mixing preformed ceramic particles with a collagen suspension [636-638]. However, in all blended composites, the crystallite sizes of calcium orthophosphates were not uniform and the crystals were often aggregated and randomly distributed within a fibrous matrix of collagen. Therefore, no structural similarity to natural bone was obtained and only a compositional similarity to that of natural bone was achieved. Crystallization of CDHA in aqueous solutions might be performed in the presence of a previously dispersed collagen [29, 500]. More to the point, collagen might be first dispersed in an acidic solution, followed by addition of calcium and orthophosphate ions and then co-precipitation of collagen and CDHA might be induced by either increasing the solution pH or adding mixing agents [37]. Although it resulted in biocomposites with poor mechanical properties, pressing of the HA/collagen mixtures at 40 °C under 200 MPa for several days is also known [639]. Attempts have been performed for a computer simulation of apatite/collagen composite formation process [640]. It is interesting to note, that collagen/HA biocomposites were found to possess some piezoelectric properties [641].

As the majority of the collagen/HA biocomposites are conventionally processed by anchoring micro-HA particles into collagen matrix, it makes quite difficult to obtain a uniform and homogeneous composite graft. Besides, such biocomposites have inadequate mechanical properties; over and above, the proper pore sizes have not been achieved either. Further, microcrystalline HA, which is in contrast to nanocrystalline natural bone apatite, might take a longer time to be remodeled into a new bone tissue upon the implantation. In addition, some of the biocomposites exhibited very poor mechanical properties, probably due to a lack of strong interfacial bonding between the constituents. The aforementioned data clearly demonstrate that the chemical composition similar to bone is insufficient for manufacturing the proper bone grafts; both the mechanical properties and mimetic of the bone nanostructure are necessary to function as bone in recipient sites. There is a chance for improving osteointegration by reducing the grain size of HA crystals by activating of ultrafine apatite growth into the matrix. This may lead to enhance the mechanical properties and osteointegration with improved biological and biochemical affinity to the host bone. Besides,

the unidirectional porosity was found to have a positive influence on the ingrowth of the surrounding tissues into the pores of collagen/HA biocomposites [642].

Bovine collagen might be mixed with HA and such biocomposites are marketed commercially as bone-graft substitutes those further can be combined with bone marrow aspirated from the iliac crest of the site of the fracture. Application of these materials was compared with autografts for the management of acute fractures of long bones with defects, which had been stabilized by internal or external fixation [643,644]. These biocomposites are osteogenic, osteoinductive and osteoconductive; however, they lack the structural strength and require harvest of the patient's bone marrow. Although no transmission of diseases has been recorded yet, the use of bovine collagen might be a source of concern [2].

Collagen sponges with an open porosity (30–100 μm) were prepared by a freeze-drying technique and then their surface was coated by a 10- μm layer of biomimetic apatite precipitated from simulated body fluid [645]. The researchers found a good *in vitro* performance with fibroblast cell culture. Collagen/HA microspheres or gel beads have been prepared in the intention of making injectable bone fillers [646, 647]. Liao *et al.* succeeded in mimicking the bone structure by blending carbonateapatite with collagen [648]. A similar material (mineralized collagen) was implanted into femur of rats and excellent clinical results were observed after 12 weeks [649]. Collagen/HA biocomposites were prepared and their mechanical performance was increased by cross-linking the collagen fibers with glutaraldehyde [501, 503, 504]. These biocomposites were tested in rabbits and showed a good biological performance, osteoconductivity and biodegradation. A similar approach was selected to prepare HA/collagen microspheres (diameter $\sim 5 \mu\text{m}$) by a water-oil emulsion technique in which the surface was also cross-linked by glutaraldehyde [647]. That material showed a good *in vitro* performance with osteoblast cell culture. A porous bone graft substitute was formed from a nano-HA/collagen biocomposite combined with PLA by a freeze-drying method; the resulting material was found to mimic natural bone at several hierarchical levels [511]. Subsequent *in vitro* experiments confirmed a good adhesion, proliferation and migration of osteoblasts into this composite [510]. A further increase in biocompatibility might be achieved by the addition of silicon; thus, to enhance bone substitution, Si-substituted HA/collagen composites have been developed with silicon located preferentially in the collagen phase [502]. Porous (porosity level $\sim 95\%$ with interconnected pores of 50–100 μm) biocomposites of collagen (crosslinked with glutaraldehyde) and β -TCP have been prepared by a freeze-drying technique, followed by sublimation of the solvent; the biocomposites showed a good biocompatibility upon implantation in the rabbit jaw [650].

Biocomposites of calcium orthophosphates with collagen were found to be useful for drug-delivery purposes [520, 608, 651-653]. Namely, an HA/collagen–alginate (20 μl) with the rh-BMP2 (100 $\mu\text{g/ml}$, 15 μl) showed bone formation throughout the implant 5 weeks after implantation without obvious deformation of the material [520]. Gotterbarm *et al.* developed a two-layered collagen/ β -TCP implant augmented with chondral inductive growth factors for repair of osteochondral defects in the trochlear groove of minipigs. This approach might be a new promising option for the treatment of deep osteochondral defects in joint surgery [652].

To conclude this part, one should note that biocomposites of apatites with collagen are a very hot topic of the research and up to now, just a few papers are devoted to biocomposites of other calcium orthophosphates with collagen [652, 654]. These biomaterials mimic natural bones to some extent, while their subsequent biological evaluation suggests that they are readily incorporated into the bone metabolism in a way similar to bone remodeling, instead of

acting as permanent implant [511, 617]. Collagraft[®], Bio-Oss[®] and Healos[®] are several examples of the commercially available calcium orthophosphate/collagen bone grafts for clinical use [32]. However, the performance of these biocomposites depends on the source of collagen from which it was processed. Several attempts have been made to simulate the collagen-HA interfacial behavior in real bone by means of crosslinking agents such as glutaraldehyde [501, 503, 504, 622, 647, 650] with the purpose to improve the mechanical properties of these biocomposites. Unfortunately, a further progress in this direction is restricted by a high cost, difficulty to control cross-infection, a poor definition of commercial sources of collagens, as well as by a lack of an appropriate technology to fabricate bone-resembling microstructures. Further details on calcium orthophosphate/collagen composites, including the list of the commercially available products, might be found elsewhere [32, 612].

4.5. Biocomposites with Other Bioorganic Compounds and Biological Macromolecules

Besides collagen, both human and mammalian bodies contain dozens types of various bioorganic compounds, proteins and biological macromolecules. The substantial amounts of them potentially might be used to prepare biocomposites with calcium orthophosphates. For example, a biologically strong adhesion (to prevent invasion of bacteria) between teeth and the surrounding epithelial tissues is attributed to a cell-adhesive protein, laminin [655]. In order to mimic the nature, a laminin/apatite biocomposite layer was successfully created on the surface of both titanium [656] and EVOH [657, 658] using the biomimetic approach.

Calcium orthophosphate/gelatin biocomposites are widely investigated as potential bone replacement biomaterials [255, 273-275, 367-375, 386-393, 403, 430-432, 457, 521-526, 659-670]. For example, gelatin foams were successfully mechanically reinforced by HA and then crosslinked by a carbodiimide derivative [255]. Such foams were shown to be a good carrier for antibiotic tetracycline [663]. Several biocomposites of calcium orthophosphates with alginates¹¹ have been prepared [390, 519, 520, 524, 596, 671]. For example, porous HA/alginate composites based on hydrogels were prepared both biomimetically [596] and by using a freeze-drying technique [671]. Another research group succeeded in preparation of biphasic but monolithic scaffolds using a similar preparation route [672]. Their biocompatibility in cell culture experiments and *in vitro* biodegradability were high; however, a mechanical strength could be better.

Various biocomposites of calcium orthophosphates with chitosan [240, 398, 413, 420, 436, 468, 546-550, 567, 568, 578, 583, 664, 670, 673-684] and chitin [184, 395, 514, 685-689] are also very popular. For example, a solution-based method was developed to combine HA powders with chitin, in which the ceramic particles were uniformly dispersed [685, 686]. Unfortunately, it was difficult to obtain the uniform dispersions. The mechanical properties of the final biocomposites were not very good; due to a poor adhesion between the filler and the matrix both the tensile strength and modulus were found to decrease with increase of the HA amount. Microscopic examination revealed that HA particles were intervened between the polymer chains, weakening their interactions and decreasing the entire strength [685, 686].

¹¹ Alginates are a family of unbranched binary copolymers with a structure comprising 1-4 glycosidically linked β -D-mannuronic acid and its C-5 epimer α -L-guluronic acid [596].

Biocomposites of CDHA with water-soluble proteins, such as bovine serum albumin (BSA), might be prepared by a precipitation method [464, 690-693]. In such biocomposites, BSA is not strongly fixed to solid CDHA, which is useful for a sustained release. However, this is not the case if a water/oil/water interfacial reaction route has been used [251]. To extend this subject, inclusion of DNA into CDHA/BSA biocomposites was claimed [251, 694-696]. Besides, bionanocomposites of an unspecified calcium orthophosphate with DNA were prepared as well [697].

Akashi and co-workers developed a procedure to prepare calcium orthophosphate-based biocomposites by soaking hydrogels in supersaturated by Ca^{2+} and PO_4^{3-} ions solutions in order to precipitate CDHA in the hydrogels (up to 70% by weight of CDHA could be added to these biocomposites) [698]. This procedure was applied to chitosan; the 3D shape of the resulting biocomposite was controlled by the shape of the starting chitosan hydrogel [699]. Another research group developed biocomposites based on *in situ* calcium orthophosphate mineralization of self-assembled supramolecular hydrogels [700].

Various biocomposites of CDHA with glutamic and aspartic amino acids, as well as poly-glutamic and poly-aspartic amino acids have been prepared and investigated by Bigi *et al.* [280, 281, 701-704]. These (poly)amino acids were quantitatively incorporated into CDHA crystals, provoking a reduction of the coherent length of the crystalline domains and decreasing the crystal sizes. The relative amounts of the (poly)amino acid content in the solid phase, determined through HPLC analysis, increased with their concentration in solution up to a maximum of about 7.8 wt.% for CDHA/aspartic acid and 4.3 wt.% for CDHA/glutamic acid biocomposites. The small crystal dimensions, which implied a great surface area, and the presence of (poly)amino acids were suggested to be relevant for possible application of these biocomposites for hard tissues replacement [280, 281, 701-704].

Recently, BCP ($\text{HA}+\beta\text{-TCP}$)/agarose macroporous scaffolds with controlled and complete interconnection, high porosity, thoroughly open pores and tailored pore size were prepared for tissue engineering application [705, 706]. Agarose, a biodegradable polymer, was selected as the organic matrix because it was a biocompatible hydrogel, which acted as gelling agent leading to strong gels and fast room temperature polymerization. Porous scaffolds with the designed architecture were manufactured by combining a low temperature shaping method with stereo-lithography and two drying techniques. The biocompatibility of this BCP/agarose system was tested with mouse L929 fibroblast and human Saos-2 osteoblast during different colonization times [705].

Fibrin sealants are non-cytotoxic, fully resorbable, biological matrices that simulate the last stages of a natural coagulation cascade, forming a structured fibrin clot similar to a physiological clot [707]. Biocomposites of calcium orthophosphates with fibrin sealants might develop the clinical applications of bone substitutes. The 3D mesh of fibrin sealant interpenetrates the macro- and micro-porous structure of calcium orthophosphate ceramics [9]. The physical, chemical and biological properties of calcium orthophosphate bioceramics and the fibrin glue might be cumulated in biocomposites, suitable for preparation of advanced bone grafts [708-719].

Furthermore, there are biocomposites of calcium orthophosphates with bisphosphonates [720], silk fibroin (that is a hard protein extracted from silk cocoon) [250, 563-565, 570, 571, 721-726], chitosan + silk fibroin [727], fibronectin [728] and casein phosphopeptides [729]. Besides, the reader's attention is pointed out to an interesting approach to crystallize CDHA inside poly(allylamine)/poly(styrene sulfonate) polyelectrolyte capsules resulting in empty

biocomposite spheres of micron size [730]. Depending on the amount of precipitated CDHA, the thickness of the shell of biocomposite spheres can be varied between 25 and 150 nm. These biocomposite capsules might find application as medical agents for bone repairing and catalytic microreactors [730].

4.6. Injectable Bone Substitutes (IBS)

Injectable bone substitutes (IBS) represent ready-to-use suspensions of calcium orthophosphate powder(s) in a liquid carrier phase. They look like viscous pastes with the rheological properties, sufficient to inject them into bone defects by means of surgical syringes and needles. Usually, the necessary level of viscosity is created by addition of water-soluble polymers [104, 731, 732]. Therefore, the majority of calcium orthophosphate-based IBS formulations might be considered as a subgroup of calcium orthophosphate/polymer biocomposites. For example, an IBS was described that involved a silanized hydroxyethylcellulose carrier with BCP, consisting of HA and β -TCP [733]. The suspension is liquid at pH within 10-12, but gels quickly at pH < 9. Injectable composites can be formed with β -TCP to improve mechanical integrity [454]. Similarly, Bennett *et al.* showed that a polydioxanone-co-glycolide-based biocomposite reinforced with HA or β -TCP can be used as an injectable or moldable putty [734]. During the crosslinking reaction following injection, carbon dioxide is released allowing the formation of interconnected pores.

Daculsi *et al.* developed viscous IBS biocomposites based on BCP (60% HA + 40% β -TCP) and 2% aqueous solution of HPMC that was said to be perfectly biocompatible, resorbable and easily fitted bone defects (due to an initial plasticity) [84, 732, 735-741]. The best ratio BCP/HPMC aqueous solution was found to be at $\sim$ 65/35 w/w. To extend this subject further, this type of IBS might be loaded by cells [742] or by microparticles [743].

The advanced characteristics of IBS come from their good mechanical properties and biocompatibility and the ease of tissue regeneration. Although the fabrication of IBS biocomposites in most cases improved the mechanical properties of the system and provided the material with resistance to fluids penetration, these achievements were limited by the amount of polymer that can be added to the paste. For instance, Mickiewicz *et al.* reported that after a critical concentration (that depended on the type and molecular weight of the polymer, but was always around 10%), the polymer started forming a thick coating on the crystal clusters, preventing them from interlocking, originating plastic flow and, as a consequence, decreasing mechanical properties [464]. More to the point, Fujishiro *et al.* reported a decrease in mechanical properties with higher amounts of gel, which was attributed to formation of pores due to leaching of gelatin in solution [457]. Therefore, it seems that mechanical properties, although improved by the addition of polymers, are still a limitation for the application of calcium orthophosphate-based IBS formulations in load-bearing sites [147].

4.7. Biocomposites with Glasses, Inorganic Materials and Metals

To overcome the problem of poor mechanical properties of calcium orthophosphate bioceramics, suitable biocomposites of calcium orthophosphates reinforced by various inorganic materials, glasses and metals have been developed. Such biocomposites are mainly

prepared by the common ceramic processing techniques such as thermal treatment after kneading [744-746], powder slurry coating [747] and metal-sol mixing [748]. For example, HA was combined with Bioglass[®] (Novabone Products, Alachua, FL) [749,750] and with other glasses [751] to form glass-ceramics biocomposites. Other reinforcement materials for calcium orthophosphates are differentiated by either shape of the fillers, namely, particles [752, 753], platelets [754, 755], whiskers [485, 756, 757], fibers [758-760], or their chemical composition: zirconia and/or PSZ [251, 744-747, 756, 761-794], alumina [251, 752, 755, 794-803], titania [308,748,753,804-818], other oxides [819-822], silica and/or glasses [823-830], wollastonite [172, 831-838], various metals and alloys [760, 795, 818, 839-852], calcium sulfate [853-855], silicon carbide [757], barium titanate [856], zeolite [857] and several other materials [272, 858-860]. All these materials have been added to calcium orthophosphate bioceramics to improve its reliability. Unfortunately, significant amounts of the reinforcing phases are needed to achieve the desired properties and, as these materials are either bioinert, significantly less bioactive than calcium orthophosphates or not bioresorbable, the ability of the biocomposites to form a stable interface with bone is poorer if compared with calcium orthophosphate bioceramics alone. Due to the presence of bioinert compounds, such formulations might be called bioinert/bioactive composites [823]. The ideal reinforcement material would impart mechanical integrity to a biocomposite at low loadings, without diminishing its bioactivity. As clearly seen from the amount of the references, apatite/zirconia biocomposites are most popular ones among the researchers.

There are several types of HA/glass biocomposites. The first one is also called bioactive glass-ceramics. A dense and homogeneous biocomposite was obtained after a heat treatment of the parent glass, which comprised ~38 wt.% oxy-FAP ($\text{Ca}_{10}(\text{PO}_4)_6(\text{O},\text{F})_2$) and ~34 wt.% β -wollastonite ($\text{CaO}\cdot\text{SiO}_2$) crystals, 50–100 nm in size in a $\text{MgO}\cdot\text{CaO}\cdot\text{SiO}_2$ glassy matrix [172,831-838]. Apatite-wollastonite (A-W) glass-ceramics is an assembly of small apatite particles effectively reinforced by wollastonite. The bending strength, fracture toughness and Young's modulus of A-W glass-ceramics are the highest among bioactive glass and glass ceramics, enabling it to be used in some major compression load bearing applications, such as vertebral prostheses and iliac crest replacement. It combines a high bioactivity with the suitable mechanical properties [861]. β -TCP/wollastonite biocomposites are also known [862-864]. More complicated biocomposites have been developed as well. For example, (A-W)/HDPE composite (AWPEX) biomaterials have been designed to match the mechanical strength of human cortical bone and to provide favorable bioactivity, with potential use in many orthopedic applications [865-868]. Other examples comprise wollastonite-reinforced HA/Ca polycarboxylate [869] and glass-reinforced HAP/polyacrylate [870] biocomposites.

HA/glass biocomposites can be prepared by simple sintering of appropriate HA/glass powder mixtures [871-874]. If sintering is carried out below 1000 °C, HA does not react with the bioactive glass [872, 873] or this reaction is limited [874]. Besides, reaction between HA and glasses depends on the glass composition. In another approach, small quantities of bioactive glass have been added to HA bioceramics in order to improve densification and/or mechanical properties [26]. In addition, biocomposites might be sintered from HA and silica [823]. In general, bioactive glass-ceramics maintain a high strength for a longer time than HA bioceramics under both the *in vitro* and *in vivo* conditions [830, 835].

Carbon nanotubes with their small dimensions, a high aspect ratio (length to diameter) as well as the exceptional mechanical properties, including extreme flexibility and strength, significant resistance to bending, high resilience and the ability to reverse any buckling of the

tube, have the excellent potential to accomplish necessary mechanical properties [875]. Recent studies have even suggested that they may possess some bioactivity [876-879]. However, due to a huge difference in shapes, it is a challenge to prepare homogeneous mixtures of calcium orthophosphates and carbon nanotubes: "one can imagine something similar to achieving a homogeneous mixture of peas and spaghetti" [875, page 7]. Additionally, non-functionalized carbon nanotubes tend to agglomerate and form bundles; besides, they are soluble in neither water nor organic solvents. Chemical functionalization allows carbon nanotubes to be dispersed more easily, which can improve interfacial bonding with calcium orthophosphates [248, 875].

Different strategies might be employed to prepare calcium orthophosphate/carbon nanotubes biocomposites. For example, apatites might be chemically synthesized by using carboxyl functionalized carbon nanotubes as a matrix [243-248]. Physico-chemical characterization of these biocomposites showed that nucleation of CDHA initiates through the carboxyl group [248]. Hot-pressing [880], plasma spraying [881] and laser surface alloying [882-884] techniques might be applied as well. The research on calcium orthophosphate (up to now, only apatites)/carbon nanotube biocomposites is in its early stages, with the first papers published in 2004 [247, 434]. Due to this reason, the mechanical property data for such biocomposites have been reported only in few papers; however, these results are encouraging. For example, Chen *et al.* performed nanoindentation tests on biocomposite coatings to give hardness and Young's modulus values [884]. They found that the higher the loading of nanotubes, the better the properties. Namely, at 20 wt.% loading, hardness was increased by 43% and Young's modulus by 21% over a single-phase HA coating [884]. Scratching test results indicated that as alloyed HA biocomposite coatings exhibited improved wear resistance and lower friction coefficient with increasing the amount of carbon nanotubes in the precursor material powders [883]. Additionally, measurements of the elastic modulus and hardness of the biocomposite coatings indicated that the mechanical properties were also affected by the amount of carbon nanotubes [882]. Another research group performed compression tests on bulk HA/nanotubes biocomposites and found an increase in strength over single-phase HA [247]. However, the highest compressive strength they achieved for any material was only 102 MPa, which is similar to that of cortical bone but much lower than the typical values for dense HA [875]. More complex formulations, such as poly-L-lysine/HA/carbon nanotube hybrid nanocomposites, have been also developed [885]. Unfortunately, carbon nanotubes are very stable substances; they are neither bioresorbable nor biodegradable. Therefore, during the *in vivo* bioresorption, the nanotubes will get into the human body from the biocomposite matrix and might cause uncertain health problems. Except of carbon nanotubes, carbon fibers of microscopic dimensions are also used to reinforce HA bioceramics [886-888].

The main disadvantage of HA reinforced by PSZ is degradation of zirconia in wet environments [756, 761, 762, 784]. Transformation of the tetragonal ZrO_2 to the monoclinic phase on the surface results in formation of microcracks and consequently lowers the strength of the implant [889, 890].

An HA-based biocomposite reinforced with 20 vol. % of Ti particles was fabricated by hot pressing [841]. Besides, calcium orthophosphates/Ti biocomposites might be prepared by powder metallurgy processing [843-845]. At high temperatures, the presence of Ti metal phase was found to promote dehydration and decomposition of HA into β -TCP and TTCP [841, 843] or partial formation of β -TCP and calcium titanate instead of HA [555, 844, 845].

Comparing with pure HA bioceramics manufactured under the same conditions, the HA/Ti biocomposites possessed a higher fracture toughness, bending strength, work of fracture, porosity and lower elastic modulus, which is more suitable for biomedical applications. However, the mechanical properties appeared to be not high enough to use HA/Ti biocomposites in load-bearing applications. Luckily, the histological evaluations revealed that HA/Ti biocomposites could be partially integrated with newborn bone tissues after 3 weeks and fully osteointegrated at 12 weeks *in vivo* [841]. Similar findings had been earlier made for HA bioceramics reinforced by addition of silver particulates (5–30 vol. %) and subsequent sintering of the HA/Ag powder compacts [839, 840]. Other studies on calcium orthophosphate/Ti biocomposites are available elsewhere [846–849].

To conclude this part, biocomposites consisting of calcium orthophosphates only should be briefly described. First of all, BCP itself, consisting of HA and α - or β -TCP, should be mentioned [23]. In 1980-s, BCP was called as “TCP ceramics complexed with HA” [891]. More to the point, 70% HA-powder + 30% HA-whisker biocomposites have been fabricated by pressureless sintering, hot pressing and hot isostatic pressing. These biocomposites were found to exhibit an improved toughness, attaining the lower fracture-toughness limit of bone without a decrease of bioactivity and biocompatibility [892, 893]. Besides, a dual HA biocomposite that combined two HA materials with different porosities: HA with 75% porosity, for bone ingrowth and HA with 0% porosity, for load bearing was manufactured. This dual HA biocomposite appeared to be suitable for use as an implant material for spinal interbody fusion as a substitute for iliac bone grafts, which could eliminate the disadvantages associated with autograft harvesting [894]. A biodegradable nanocomposite porous scaffold comprising a β -TCP matrix and HA nanofibres was developed and studied for load-bearing bone tissue engineering. HA nanofibres were prepared by a biomimetic precipitation method, the inclusion of which significantly enhanced the mechanical property of the scaffold, attaining a compressive strength of 9.87 MPa, comparable to the high-end value (2–10 MPa) of cancellous bone [895].

4.8. Functionally Graded Biocomposites

Although, in most cases, the homogeneous distribution of filler(s) inside a matrix is required [356], there are composites, where this is not the case. For example, functionally graded materials (commonly referred to as FGM) might be characterized by the intentional variations in composition and/or structure gradually over volume, resulting in corresponding changes in the properties of the composite. The main feature of such materials is the almost continuously graded composition that results in two different properties at the two ends of the structure. Such composites can be designed for specific function and applications. Various approaches based on the bulk (particulate processing), preform processing, layer processing and melt processing are used to fabricate the functionally graded materials.

Bone is a biologically formed composite with variable density ranging from very dense and stiff (the cortical bone) to a soft and foamed structure (the trabecular bone). Normally the outer part of long bones consists of cortical bone with the density decreasing towards the core, where the trabecular bone is found. The trabecular bone is porous and the porosity is filled with osseous medulla [21, 22]. This brief description clearly indicates that bones are natural functionally graded composites.

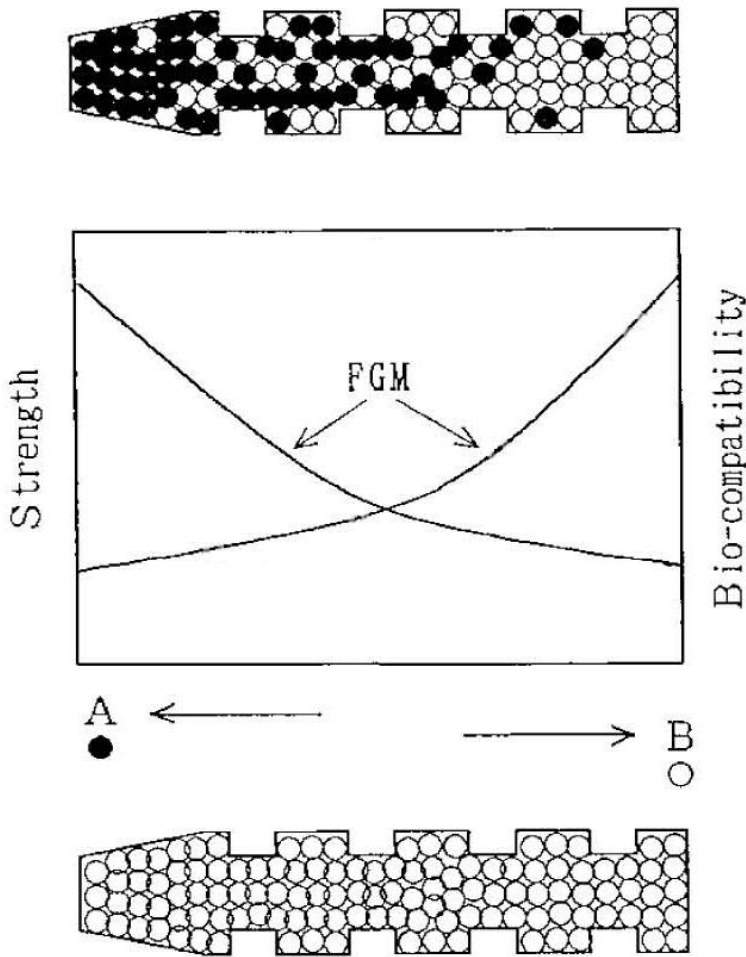


Figure 3. Expected properties of functionally graded biocomposite dental implant. For comparison, the upper drawing shows a functionally graded implant and the lower one shows a conventional uniform implant. The properties are shown in the middle. The implant with the composition changed from a biocompatible metal (Ti) at one end (left in the figure), increasing the concentration of bioceramics (HA) toward 100% HA at the other end (right in the figure), could control both mechanical properties and biocompatibility without an abrupt change due to the formation of discrete boundary. This FGM biocomposite was designed to provide more titanium for the upper part where occlusal force is directly applied and more HA for the lower part, which is implanted inside the jawbone. Reprinted from Ref. [897] with permission.

The concept of FGM has been increasingly used for biomaterial design and currently it remains to be an important area of the research. For example, powder metallurgy methods have been used to fabricate HA/Ti functionally graded biocomposite dental implants offering the biocompatible HA on the tissue side and titanium on the outer side for mechanical strength [896-898]. The graded structure in the longitudinal direction contains more Ti in the upper section and more HA in the lower section. Actually, in the upper section the occlusal force is directly applied and Ti offers the required mechanical performance; in the lower part, which is implanted inside the bone, the HA confers the bioactive and osteoconductive properties to the material [896]. Since the optimum conditions of sintering for Ti and HA are

very different, HA/Ti functionally graded biocomposites are difficult to fabricate and the sintering conditions for their mixtures are obliged to compromise. The expected properties of this implant are shown in Fig. 3 [897]. Functionally graded HA/Ti biocomposite coatings might be prepared by rf-plasma spraying [899]. A functionally graded HA/PMMA biocomposite was developed based on sedimentary HA distributions in a PMMA viscous fluid, using a centrifuge to avoid stress convergence on the interface. The stress-strain curves of this biocomposite showed sufficient strength for medical application along with the relaxation of brittleness and fragility [449]. A three-layered graded biocomposite membrane, with one face of 8% nano-carbonated CDHA/collagen/PLGA porous membrane, the opposite face of pure PLGA non-porous membrane, the middle layer of 4% nano-carbonated CDHA/collagen/PLGA as the transition was prepared through the layer-by-layer casting method [513]. HA/glass FGM layers were coated on titanium alloy (Ti-6Al-4V) substrates. The design of these layers and the use of the glass were for achieving a strong bonding between the FGM layered coatings and the substrates [900, 901]. More to the point, Ti alloy substrate has been combined with HA granules spread over the surface [902].

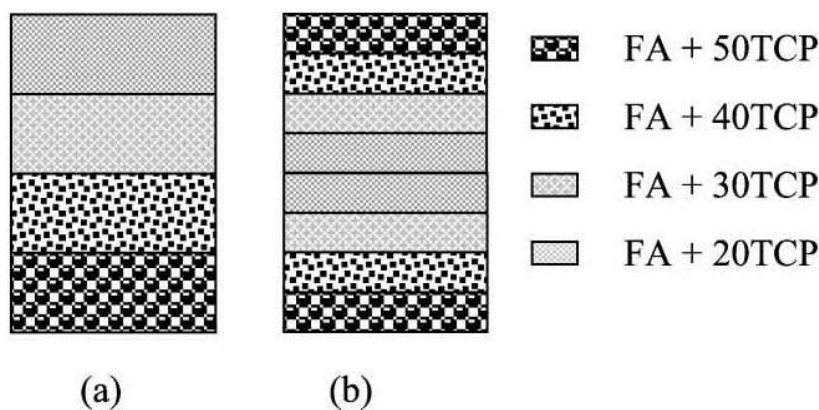


Figure 4. A schematic diagram showing the arrangement of the FA/ β -TCP composite layers: (a) non-symmetric FGM, (b) symmetric FGM. Reprinted from Ref. [903] with permission.

Functionally graded β -TCP/FA biocomposites combine the biostability of FA with bioresorbable properties of β -TCP [903]. An interesting multilayered (each layer of 1 mm thick) structure consisting of β -TCP/FA biocomposites with different molar ratios has been prepared, giving rise to formation of a FGM (Fig. 4). After implantation, the preferential dissolution of β -TCP phase would result in functionally gradient porosity for bone ingrowth [904]. HA/zirconia graded biocomposites were fabricated to enhance the mechanical properties of HA while retaining its bone bonding property [792]. TiO₂ and HA were found to be a good combination for FGM providing both a gradient of bioactivity and a good mechanical strength [904]. Besides, graded HA/CaCO₃ biocomposite structures for bone ingrowth have been developed as well [905]. Functionally graded composite skull implants consisting of polylactides, carbonateapatite and CaCO₃ are known as well [319,320]. The research in this field is quite promising but currently the mechanical properties of the available biocomposites are clearly in excess of the properties of bone [148].

4.9. Biosensors

A biosensor is a device for detection of an analyte that combines a biological component with a physicochemical detector component. Very briefly, it consists of 3 parts: a sensitive biological element; a transducer or a detector element that transforms the signal resulting from the interaction of the analyte with the biological element into another signal; and associated electronics that is primarily responsible for the display of the results in a user-friendly way [906].

The surface of biologically relevant calcium orthophosphates (CDHA, HA, α -TCP, β -TCP) has an excellent ability of adsorption for functional biomolecules such as proteins, albumins, DNA and so on. Therefore, some calcium orthophosphate-based biocomposites and hybrid biomaterials were found to be applicable for biosensor manufacturing [289, 543, 852, 885]. For example, formation of poly-L-lysine/HA/carbon nanotube hybrid nanoparticles was described and a general design strategy for an immunosensing platform was proposed based on adsorption of antibodies onto this nanocomposite [885]. In another paper, a hybrid material formed by assembling of gold nanoparticles onto nano-HA was employed for the interface design of piezoelectric immunosensor, on which the antibodies were bound. The developed sensing interface appeared to possess some advantages, such as activation-free immobilization and high antigen-binding activities of antibodies, over using either nano-HA or gold nanoparticles alone [852]. Up to date, just a few papers have been published on biosensor application of calcium orthophosphate-based biocomposites. Presumably, this subject will be further developed in the future and, perhaps, sometime implantable biosensors will be designed to perform the continuous concentration monitoring of the important biological macromolecules. Possibly, those biocensors might be able to use an electric power, generated by DCPD/polymer composite-based battery devices [414, 415].

INTERACTION BETWEEN THE PHASES IN CALCIUM ORTHOPHOSPHATE-BASED BIOCOMPOSITES

An important aspect that should be addressed in details is a mutual interaction between calcium orthophosphates and other phases in biocomposites and hybrid biomaterials. In general, an interaction between the phases in any composite can be either mechanical, when it results from radial compression forces exerted by the matrix on the filler particles (for example, developed during cooling due to thermal contraction), or chemical, when the reactivity of the filler towards the matrix has an important role. In the latter case, it is important to distinguish a physical interaction from chemical bonding [226]. According to Wypych [907], physical interaction is more or less temporary, implicating hydrogen bonding or van der Waals forces, whereas chemical bonding is stronger and more permanent, involving covalent bond formation. Thus, a chemical interfacial bond between the phases is preferred to achieve a higher strength of a composite. The magnitude of the interfacial bond between the phases determines how well a weak matrix transmits stress to the strong fibers. However, while a bond between the matrix and reinforcement must exist for the purpose of stress transfer, it should not be so strong that it prevents toughening mechanisms, such as debonding and fiber pullout [875].

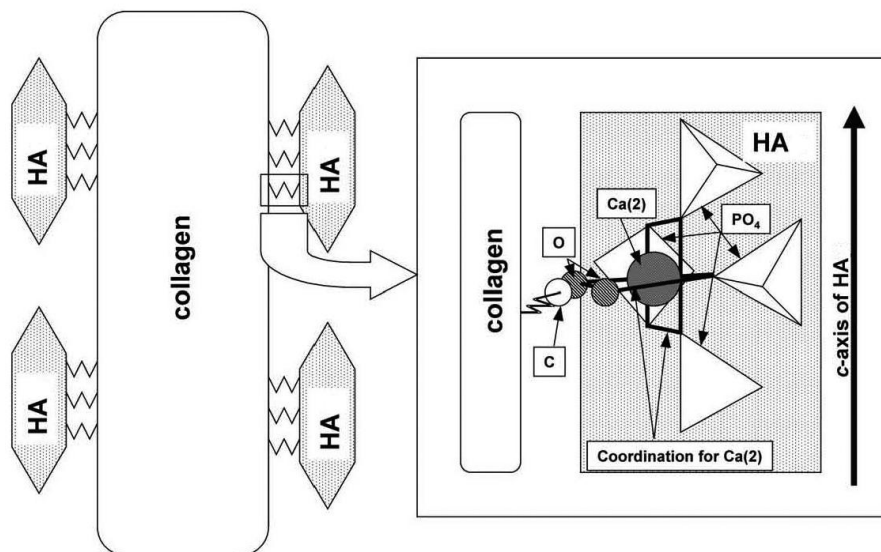


Figure 5. A schematic drawing of the relation between self-organization (directional deposition of HA on collagen) and interfacial interaction in biocomposites. Direction of interaction between HA and collagen is restricted by covalent bond between COO and Ca(2) to maintain regular coordination number of 7. Reprinted from Ref. [629] with permission.

There is still doubt as to the exact bonding mechanism between bone minerals (biological apatite) and collagen, which undoubtedly plays a critical role in determining the mechanical properties of bones. Namely, bone minerals are not directly bonded to collagen but through non-collagenous proteins that make up ~3 % of bones (Table 1) and provide with active sites for biomineralization and for cellular attachment [32]. In bones, the interfacial bonding forces are mainly ionic bonds, hydrogen bonds and hydrophobic interactions, which give the bones the unique composite behavior [49]. There is an opinion that, opposite to bones, there is no sign of chemical bonding between phases in conventional calcium orthophosphate/collagen biocomposites, probably due to a lack of suitable interfacial bonding during mixing [35]. However, this is not the case for phosphorylated collagens [634]. Anyway, Fourier-transformed infrared (FTIR) spectra of some calcium orthophosphate-based composites and collagen films were measured and transformed into absorption spectra using the Kramers-Kronig equation to demonstrate energy shifts of residues on the HA/collagen interface. After comparing FTIR spectra of biocomposites and collagen films in detail, red shifts of the absorption bands for C–O bonds were observed in the spectra of the biocomposites. These red shifts were described as a decrease of bonding energies of C–O bonds and assumed to be caused by an interaction to Ca^{2+} ions located on the surfaces of apatite nanocrystals, as shown in Fig. 5 [629]. Another proof of a chemical interaction between CDHA and collagen fibers was also evaluated in FTIR spectra of CDHA/collagen biocomposites, in which a shift of the band corresponding to $-\text{COO}^-$ stretching from 1340 to 1337 cm^{-1} was observed [595,596]. More to the point, nucleation of CDHA crystals onto collagen through a chemical interaction with carboxylate groups of collagen macromolecules has been reported [908-910].

FTIR-spectroscopy seems to be the major investigation tool of a possible chemical bonding among the phases in calcium orthophosphate-based biocomposites and hybrid

biomaterials [221, 282, 288, 290, 383, 421, 503, 518, 527, 530, 537, 540, 542, 545, 550, 557, 566, 571, 596, 634, 667, 668, 727, 911, 912]. For example, the characteristic bands at 2918, 2850 and 1472 cm^{-1} for the hydrocarbon backbone of PE appeared to have zero shift in an HA/PE biocomposite. However, in the case of polyamide, some of the FTIR-bands indicated that the polar groups shifted apparently: the bands at 3304, 1273 and 692 cm^{-1} derived from stretching of N–H, stretching of C–N–H and vibrating of N–H moved to 3306, 1275 and 690 cm^{-1} in an HA/polyamide biocomposite, respectively. Both stretching (3568 cm^{-1}) and vibrating (692 cm^{-1}) modes of hydroxyl in HA moved to 3570 and 690 cm^{-1} in the HA/polyamide biocomposite respectively, indicating the formation of hydrogen bonds. Besides, the bands at 1094 and 1031 cm^{-1} of PO_4 modes also shifted to 1093 and 1033 cm^{-1} in the HA/polyamide biocomposite. The bands shift in a fingerprint area indicated that the hydroxyl and orthophosphate on the surface of HA might interact with plentiful carboxyl and amino groups of polyamide through nucleophilic addition [221]. Comparable conclusions were made for nano-HA/PVA [542], CDHA/alginate [596], ACP/PPF [421], HA/maleic anhydride [290] and β -TCP/PLLA [383] biocomposites, where a weak chemical bond was considered to form between Ca^{2+} ions located on the nano-HA, CDHA, ACP, HA or β -TCP surface, respectively, and slightly polarized O atoms of C=O bonds in the surrounding bioorganic compounds. Schematically, this chemical interaction is shown in Fig. 6 [596].

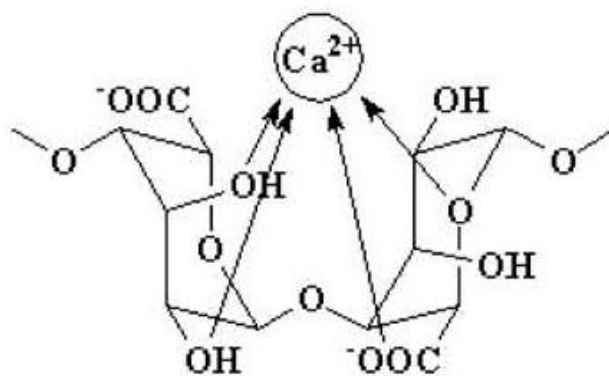


Figure 6. A schematic diagram of Ca^{2+} ion binding with alginate chains. Reprinted from Ref. [596] with permission.

Except of FTIR-spectroscopy, other measurement techniques are also able to show some evidences of a chemical interaction between calcium orthophosphates and other compounds in biocomposites [282, 383, 537, 540, 542, 912-914]. For example, for CDHA/alendronate nanocrystals such evidences were observed by thermogravimetric analysis: DTG plots of the nanocrystals appeared to be quite different from those obtained from mechanical mixtures of CDHA and calcium alendronate with similar compositions [913]. Analogous DTG results were obtained for nano-HA/PVA [542]. In the case of nano-HA/polyamide biocomposites, a hydrogen bonding between the phases was detected by differential scanning calorimetry technique [537]. Another example comprises application of the dynamic mechanical analysis to investigate softening mechanism of β -TCP/PLLA biocomposites [383]. In the case of nano-HA/PVAP composites, the indirect evidences of chemical bonding between the phases were found by X-ray diffraction and thermogravimetric analysis [282]. A strong structural

correlation between the orientation of FA crystallites and gelatin within the FA/gelatin composite spheres was discovered that indicated to a substantial reorganization of the macromolecular matrix within the area of a growing aggregate [367].

By means of the X-ray photo-electronic spectroscopy (XPS) technique, binding energies of Ca, P and O atoms were found to have some differences between nano-HA (Ca: 350.5 and 345.5; O: 530.2; P: 132.5 eV) and nano-HA/konjac glucomannan/chitosan biocomposite (Ca: 352.1 and 347.4; O: 531.2; P: 133.4 eV), respectively [550]. Further measurements by FTIR and X-ray diffraction revealed that nano-HA was mainly linked with konjac glucomannan and chitosan by hydrogen bonding among OH^- and PO_4^{3-} of nano-HA and $-\text{C}=\text{O}$ and $-\text{NH}$ of konjac glucomannan and chitosan copolymer and there was a stable interface formed between the three phases in the biocomposite. Meanwhile, coordinate bonding might be formed between Ca^{2+} and $-\text{NH}$. Stable interfaces have been formed among the three phases in a biocomposite [550]. In HA/collagen biocomposites, a covalent bond formation between Ca^{2+} of HA and RCOO^- of collagen molecules was found by XPS [504]. Similar XPS observations were also made for several other calcium orthophosphate-based biocomposites [530, 557, 566].

The interaction and adhesion between calcium orthophosphate fillers and respective matrixes have a significant effect on the properties of particulate filled reinforced materials, being essential to transfer the load between the phases and thus improve the mechanical performance of the composites [288]. However, for the substantial amount of the discussed in this book biocomposites, the interaction between the phases is mechanical in nature. This is because the matrix often consists of compounds with no functional groups or unsaturated bonds, which can form ionic complexes with the constituents of calcium orthophosphates. Obviously, less coupling exists between non-polar polymers and calcium orthophosphate ceramic particles. Therefore, polymers with functional groups pendant to the polymer backbone, which can act as sites for bridging to calcium orthophosphates, are more promising in this respect [49]. Besides, the surface of calcium orthophosphates might be modified as well [116, 417, 418, 553, 915, 916]. In order to improve the situation, various supplementary reagents are applied. Namely, if the primary effect of a processing additive is to increase the interaction between the phases, such an additive can be regarded as a coupling agent [917]. Coupling agents establish chemical bridges between the matrix and the fillers, promoting the adhesion between the phases. In many cases, their effect is not unique, influencing also the rheology of composites [226].

Optimization of biocomposite properties with coupling agents is currently an important area of the research. The control and development of molecular-level associations of polymer with calcium orthophosphates is suggested to be significant for the resulting mechanical responses in the composites. It appears that a fundamental molecular understanding of interfacial behavior in biocomposite systems is an area not sufficiently addressed in literature. Various experimental characterization techniques using electron microscopy, vibrational spectroscopy, X-ray diffraction, scanning probe microscopy and others are used routinely to characterize these materials besides mechanical property characterization. In addition, atomic scale models for simulating the phase interaction and predicting responses in the novel material systems, where nanostructure and nanointerfaces are included, are important to understand and predict the load deformation behavior [148].

A hexamethylene diisocyanate coupling agent was used to bind PEG/PBT (PolyactiveTM) block copolymers [235] and other polymers [911] to HA filler particles. Thermogravimetric

and infrared analysis demonstrated that the polymers were chemically bonded to the HA particles through the isocyanate groups, making it a suitable approach to improve the adhesion [911]. Other researchers used glutaraldehyde as a cross-linked reagent in various calcium orthophosphate-based biocomposites [389, 393, 501, 503, 504, 521, 526, 586, 622, 647, 650, 918]. The interfacial bonding between calcium orthophosphates and other components might be induced by using various coupling agents and surface modifiers, such as silanes [193, 235, 338, 541, 919-924], zirconates [226, 338, 340, 915, 925], titanates [226, 338, 925], phosphoric acid [544], alkaline pretreatment [723, 726], polyacids [115, 116, 235] and other chemicals. Besides, some polymers might be grafted onto the surface of calcium orthophosphates [553]. Structural modifications of the polymeric matrices, for instance, with introduction of acrylic acid [196, 235, 920, 921], have also proved to be effective methods. For example, application of polyacids as a bonding agent for HA/Polyactive™ composites caused the surface modified HA particles to maintain better contact with the polymer at fracture and improved mechanical properties [115, 116, 235]. The use of titanate and zirconate coupling agents appeared to be very dependent on the molding technique employed [226]. Silane-coupled HA powders were tested before applying them as fillers in biodegradable composites [922-924]. This treatment allowed HA withstanding the attack of water without impairing overall bioactivity. Besides, chemically modified reinforcement phase-matrix interface were found to improve the mechanical properties of the biocomposites. Examples of such interface modified biocomposites include chemically coupled HA/PE [920, 921], chemically formed HA/Ca poly(vinylphosphonate) [284] and PLA/HA fibers [185]. These biocomposites are able to consume a large amount of energy in the fracture.

The action of some coupling agents was found to combine two distinct mechanisms: (i) crosslinking of the polymeric matrix (valid for zirconate and titanate coupling agents) and (ii) improvement of the interfacial interactions between the major phases of the composites. This interfacial adhesion improvement appeared to be much dependent on the chemical nature (pH and type of metallic centre) of the coupling agents [338]. Several works claimed that silanes do interact with HA [193, 920-924]. It was shown that a silicon-containing inter-phase existed between HA and PE, which promoted the chemical adhesion between the HA particles and the polymer. A silane-coupling agent also facilitated penetration of PE into cavities of individual HA particles, which resulted in enhanced mechanical interlocking at the matrix-reinforcement interface [920, 921].

Addition of adhesion promoting agents might be an alternative to improve the interaction between the fillers and the matrix. For example, Morita *et al.* used incorporation of 4-methacryloyloxyethyl trimellitate anhydride to promote adhesion of the polymer to HA [926]. In another study, phosphoric ester was added to the liquid component of the formulation [927]. Both the strength and the affinity index of biocomposites were found to increase, probably due to the effects of co-polymerization.

Possible interactions between BCP and HPMC have been investigated in IBS composites [737, 738, 928]. After mixing, there was a decrease in the mean diameter of BCP granules and this influenced the viscosity of the paste. Dissolution of grain boundaries of β -TCP crystals and precipitation of CDHA on HA crystal surface was found during the interaction between BCP and HPMC in aqueous solutions. Both phenomena were responsible for the observed granulometric changes [737, 738]; however, within the sensitivity of the employed measurement techniques, no chemical bonding between BCP and HPMC was detected [928].

A co-precipitation method was used to prepare CDHA/chitosan biocomposites [673]. Growth of CDHA crystals was inhibited by organic acids with more than two carboxyl groups, which strongly bind to CDHA surfaces via a COO–Ca bond. Transmission electron microscopy images revealed that CDHA formed elliptic aggregates with chemical interactions (probably coordination bond) between Ca on its surface and amino groups of chitosan; the CDHA nanocrystals were found to align along the chitosan molecules, with the amino groups working as the nucleation sites [673]. Formation of calcium cross-linked polymer carboxylate salts was suggested during setting of calcium orthophosphate cement (TTCP+DCPA)/polyphosphazane biocomposites; the chemical involvement of the polymer in the cement setting was concluded based on the results of pH monitoring [461-463].

A chemical bond between the phases was presumed in PCL/HA composites, prepared by the grafting technique [351]; unfortunately, no strong experimental evidences were provided. In another study, CDHA/poly(α -hydroxyester) composites were prepared by a low temperature chemical route [325]. In that study, pre-composite structures were prepared by combining α -TCP with PLA, PLGA and copolymers thereof. The final biocomposite structure was achieved by *in situ* hydrolysis of α -TCP to CDHA performed at 56 °C either in solvent cast or pressed pre-composites. That transformation occurred without any chemical reaction between the polymer and calcium orthophosphates, as it was determined by FTIR spectroscopy [325].

In nearly every study on HA/carbon nanotubes biocomposites, the nanotubes have been functionalized before combining them with HA. Most researchers have done this by oxidation [243-247], although non-covalent functionalizing with sodium dodecylsulfate [247] and coating the nanotubes by a polymer [929] before combining them with HA have also been reported. Several studies by transmission electron microscopy have shown evidences that the functionalization has enhanced interaction between carbon nanotubes and HA [246, 247, 930].

If calcium orthophosphate-based biocomposites are able to sustain a high-temperature sintering (valid for the formulations consisting of inorganic components only), an inter-diffusion of chemical elements will take place between the phases. Such effect has been detected by energy-dispersive X-ray spectroscopy in HA/TiO₂ biocomposite particles with partial formation of calcium titanates; this process was found to be favorable to enhancing the cohesive strength of particles in the composite coating [818]. A similar high-temperature interaction between HA and zirconia [744,769], as well as between HA and Ti [555, 841, 843-845] was also detected. Besides, partial decomposition of HA and formation of different calcium aluminates were detected in HA/Al₂O₃ biocomposites after sintering at 1200-1300 °C [796, 802, 803].

BIOACTIVITY AND BIODEGRADATION OF CALCIUM ORTHOPHOSPHATE-BASED BIOCOMPOSITES

The continuous degradation of an implant causes a gradual load transfer to the healing tissue, preventing stress-shielding atrophy and stimulates the healing and remodeling of bones. Some requirements must be fulfilled by the ideal prosthetic biodegradable materials, such as biocompatibility, adequate initial strength and stiffness, retention of mechanical

properties throughout sufficient time to assure its biofunctionality and non-toxicity of the degradation by-products [147]. Generally speaking, bioactivity (*i.e.*, ability of bonding to bones) of biologically relevant calcium orthophosphates reinforced by other materials is usually lower than that of pure calcium orthophosphates [27, 28, 931].

In general, both bioactivity and biodegradability of any biocomposite are determined by the same properties of the constituents. Both processes are very multi-factorial because, after implantation, the surface of any graft is rapidly colonized by cells. Much more biology, than chemistry and material science altogether, is involved into these very complex processes and many specific details still remain unknown. To simplify the task, the biodegradability of the biologically relevant calcium orthophosphates might be described by a chemical dissolution in slightly acidic media (calcium orthophosphates are almost insoluble in alkaline solutions [87-93]), which, in the case of CDHA, might be described as a sequence of four successive chemical equations [428, 932, 933]:

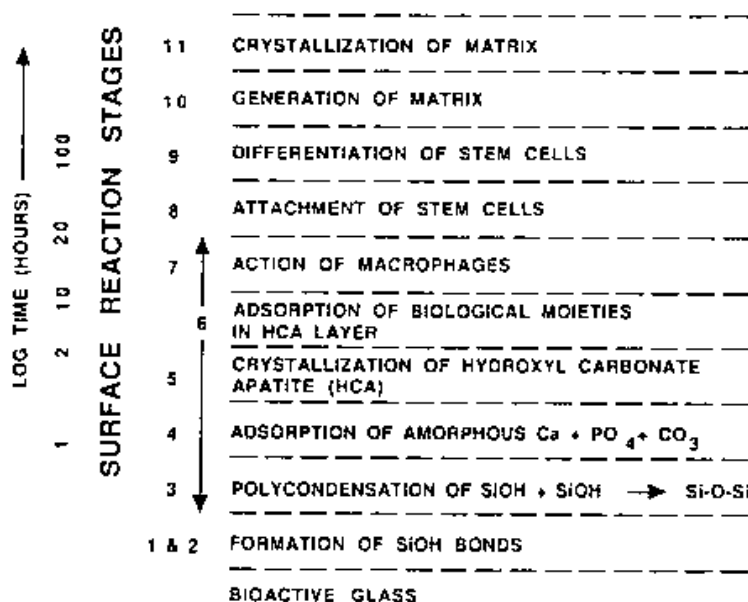
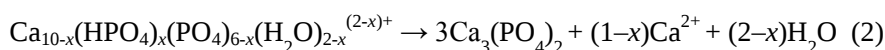
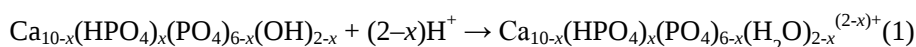


Figure 7. The sequence of interfacial reactions involved in forming a bond between tissue and bioactive glasses. The border between “dead” and “alive” occurs approximately at stage 6. For want of anything better, the bioactivity mechanism of calcium orthophosphates should also be described by this scheme with omitting of several initial stages, as it was made for HA in Ref. [72], where 3 initial chemical stages of the Hench’s mechanism were replaced by partial dissolution of HA. Reprinted from Ref. [28] with permission.

Strange enough, but the bioactivity mechanism of calcium orthophosphates is not well described in literature; therefore, biomaterials researchers [72] are forced to use a modified scheme for the bioactivity mechanism of bioactive glasses – the concept introduced by Prof. Larry Hench [27, 28]. The mechanism of bonding of bioactive glasses to living tissue involves a sequence of 11 successive reaction steps. The initial 5 steps occurred on the surface of bioactive glasses are “chemistry” only, while the remaining 6 steps belong to “biology” because the latter include colonization by osteoblasts, followed by proliferation and differentiation of the cells to form a new bone that had a mechanically strong bond to the implant surface (Fig. 7).

Biodegradability of polymers generally depends on the following factors: 1) chemical stability of the polymer backbone, 2) hydrophobicity of the monomer, 3) morphology of the polymer, 4) initial molecular weight, 5) fabrication processes, 6) geometry of the implant, 7) properties of the scaffold such as porosity and pore diameter [265]. A summary on degradation of PLA and PGA, as well as that of starch/ethylene vinyl alcohol copolymer (SEVA) is available in literature [Ref. 147, p. 798 and p. 803, respectively], where the interested readers are referred to. Biodegradation of HA/PLLA and CDHA/PLLA composite rods in subcutis and medullary cavities of rabbits were investigated mechanically and histologically; the degradation was found to be faster for the case of using uncalcinated CDHA instead of calcinated HA [934]. In a more detailed study, new bone formation was detected at 2 weeks after implantation, especially for formulations with a high HA content [935]. More to the point, a direct contact between bones and these composites without intervening fibrous tissue was detected in this case [935, 936]. SEVA-C and SEVA-C/HA biocomposites were found to exhibit a non-cytotoxic behavior [937, 938], inducing a satisfactory tissue response when implanted as shown by *in vivo* studies [938]. Furthermore, SEVA-C/HA biocomposites induce a positive response on osteoblast-like cells to what concerns cell adhesion and proliferation [937].

Both *in vitro* (the samples were immersed into 1% trypsin/phosphate-buffered saline solution at 37°C) and *in vivo* (implantation of samples into the posterolateral lumbar spine of rabbits) biodegradation have been investigated for nano-HA/collagen/PLA biocomposites [512]. The results demonstrated that weight loss increased continuously *in vitro* with a reduction in mass of 19.6% after 4 weeks. During the experimental period *in vitro*, the relative rate of reduction of the three components in this material was shown to differ greatly: collagen decreased the fastest, from 40% by weight to 20% in the composite; HA content increased from 45 to 60%; while PLA changed little. *In vivo*, the collagen/HA ratio appeared to be slightly higher near the transverse process than in the central part of the intertransverse process [512]. These data clearly demonstrate a biodegradation independence of various components of biocomposites.

SOME CHALLENGES AND CRITICAL ISSUES

The scientific information summarized in this book represents the recent developments of calcium orthophosphate-based biocomposites and hybrid biomaterials from a variety of approaches, starting from conventional ones to tissue engineering. Such formulations combined with osteoconductive, osteoinductive factors, and/or osteogenic cells have gained

much interest as a new and versatile class of biomaterials and are perceived to be beneficial in many aspects as bone grafts [32]. However, current applications of these biomaterials in medicine and surgery are still remarkably less than might be expected. In many biomedical applications, research and testing of such formulations have been introduced and highly developed but only in a very few cases an industrial production and commercial distribution of medical devices partially or entirely made of biocomposites have started. The medical application of biocomposites and hybrid biomaterials requires a better understanding of the objectives and limitations involved. Recently, the main critical issues have been summarized as follows [214]:

- There are not enough reliable experimental and clinical data supporting the long-term performance of biocomposites with respect to monolithic traditional materials.
- The design of biocomposites and hybrid biomaterials is far more complex than that of conventional monolithic materials because of the large number of additional design variables that must be considered.
- The available fabrication methods may limit the possible reinforcement configurations, may be time consuming, expensive, highly skilled and may require special cleaning and sterilization processes.
- There are no satisfactory standards yet for biocompatibility testing of the biocomposite implants because the ways in which the different components of any biocomposite interact to living tissues are not completely understood.
- There are no adequate standards for the assessment of biocomposite fatigue performance because the fatigue behavior of such materials is far more complex and difficult to predict than that of traditional materials [214].

On the other hand, in spite of an enormous progress in biocomposite processing, to achieve the desired characteristics researchers still need to develop more advanced technologies to fabricate a bone-resembling hierarchical organization over several length scales. Development of novel bone repair materials depends on the progress in research into the structure of natural bones. The key issues are not only to understand the fundamentals of biomineralization but also to translate such knowledge into practical synthetic pathways to produce better bone grafts. Unfortunately, when it comes to the fabrication of composites mimicing natural bone from the nanometer to the micrometer dimensions, there are many key issues, including control of morphology, incorporation of foreign ions, interaction with biomolecules and assembly of the organic and inorganic phases, which are still not well understood. A processing gap between the lower-level building units and the higher-order architecture could severely limit the practical application of current calcium orthophosphate-based biocomposites and hybrid biomaterials. Therefore, further substantial research efforts have been outlined to address the following key challenges [32, 37]:

- Optimizing biocomposite processing conditions.
- Optimization of interfacial bonding and strength equivalent to natural bone.
- Optimization of the surface properties and pore size to maximize bone growth.
- Maintaining the adequate volume of the construct *in vivo* to allow bone formation to take place.
- Withstanding the load-bearing conditions.

- Matching the bioresorbability of the grafts and their biomechanical properties while forming new bone.
- Understanding the molecular mechanisms by which the cells and the biocomposite matrix interact with each other *in vivo* to promote bone regeneration.
- Supporting angiogenesis and vascularization for the growth of healthy bone cells and subsequent tissue formation and remodeling [32, 37].

The aforementioned critical issues have to be solved before a widespread commercial use of calcium orthophosphate-based biocomposites and hybrid biomaterials can be made in surgery and medicine.

CONCLUSIONS

All types of calcified tissues of humans and mammals appear to possess a complex hierarchical composite structure. Their mechanical properties are outstanding (considering weak constituents from which they are assembled) and far beyond those, that can be achieved using the same synthetic materials with present technologies. This is because biological organisms produce biocomposites that are organized in terms of both composition and structure, containing both brittle calcium orthophosphates and ductile bioorganic components in very complex structures, hierarchically organized at the nano-, micro- and meso-levels. Additionally, the calcified tissues are always multifunctional: for example, bone provides structural support for the body plus blood cell formation. The third defining characteristic of biological systems, in contrast with current synthetic systems, is their self-healing ability, which is nearly universal in nature. These complex structures, which have risen from millions of years of evolution, inspire materials scientists in the design of novel biomaterials [939].

Up to now, still no reasonable alternative exists to autogenous bone grafts in surgery. However, the studies summarized in this book have shown that the proper combination of a ductile matrix with a brittle, hard and bioactive calcium orthophosphate filler offers many advantages for biomedical applications. Namely, the desirable properties of some components can compensate for a poor mechanical behavior of calcium orthophosphate bioceramics, while in turn the desirable bioactive properties of calcium orthophosphates improve those of other phases, thus expanding the possible application of each material within the body [94]. However, the reviewed literature clearly indicates that among possible types of calcium orthophosphate-based biocomposites and hybrid biomaterials only simple, complex and graded ones (see classification of the composites in chapter 2 of this book) have been investigated. Presumably, a future progress in this subject will require concentrating efforts on elaboration and development of hierarchical biocomposites. Certainly, in the nearest future, much attention will be paid to further development of nano-sized and nanocrystalline calcium orthophosphates [940]. Furthermore, following the modern tendency of tissue engineering, a novel generation of calcium orthophosphate-based biocomposites and hybrid biomaterials should also contain a biological living part.

Much work remains to be done on a long way from a laboratory to clinics and the success in this field depends on the effective co-operation of clinicians, chemists, biologists, bioengineers and materials scientists.

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NOMENCLATURE

EVOH	ethylene-vinyl alcohol copolymer
IBS	injectable bone substitute
HDPE	high-density polyethylene
HIPS	high impact polystyrene
HPMC	hydroxypropylmethylcellulose
PAA	polyacrylic acid
PBT	polybutyleneterephthalate
PCL	poly(ϵ -caprolactone)
PDLLA	poly-DL-lactic acid
PE	polyethylene
PEEK	polyetheretherketone
PEG	polyethylene glycol
PGA	polyglycolic acid
PHB	polyhydroxybutyrate
PHBV	poly(hydroxybutyrate-co-hydroxyvalerate)
PHEMA	polyhydroxyethyl methacrylate
PHV	polyhydroxyvalerate
PLA	polylactic acid
PLGA	poly(lactic-co-glycolic) acid
PLGC	co-polyester lactide-co-glycolide-co- ϵ -caprolactone
PLLA	poly(L-lactic acid)
PMMA	polymethylmethacrylate
PPF	poly(propylene-co-fumarate)
PS	polysulfone
PSZ	partially stabilized zirconia

PTFE	polytetrafluoroethylene
PVA	polyvinyl alcohol
PVAP	polyvinyl alcohol phosphate
SEVA	ethylene vinyl alcohol copolymer
UHMWPE	ultrahigh molecular weight polyethylene

Chapter 2

CALCIUM ORTHOPHOSPHATE CEMENTS AND CONCRETES

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ABSTRACT

In early 1980-s, researchers discovered self-setting calcium orthophosphate cements, which are a bioactive and biodegradable grafting material in the form of a powder and a liquid. Both phases after mixing form a viscous paste that after being implanted sets and hardens within the body as either a non-stoichiometric calcium deficient hydroxyapatite (CDHA) or brushite, sometimes blended with unreacted particles and other phases. As both CDHA and brushite are remarkably biocompatible and bioresorbable (therefore, in vivo they can be replaced with a newly forming bone), calcium orthophosphate cements represent a good correction technique of non-weight-bearing bone fractures or defects and appear to be very promising materials for bone grafting applications. Besides, these cements possess an excellent osteoconductivity, molding capabilities and easy manipulation. Nearly perfect adaptation to the tissue surfaces in bone defects and a gradual bioresorption followed by new bone formation are additional distinctive advantages of calcium orthophosphate cements. Furthermore, reinforced cement formulations are available, which in a certain sense might be described as calcium orthophosphate concretes. The concepts established by calcium orthophosphate cement pioneers in the early 1980-s were used as a platform to initiate a new generation of bone substitute materials for commercialization. Since then, advances have been made in the composition, performance and manufacturing; several beneficial formulations have already been introduced as a result. Many other compositions are in experimental stages. In this review, an insight into calcium orthophosphate cements and concretes, as excellent biomaterials suitable for both dental and bone grafting application, has been provided.

Keywords: calcium orthophosphate, cement, concrete, self-setting, hydraulic, injectable, bone grafting, dental, reinforced, calcium deficient hydroxyapatite, brushite, bioceramics, biomaterials, osteoconductivity, bioresorbability, materials science.

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1. INTRODUCTION

Calcium orthophosphates have been studied as bone repair materials for the last 80 years. The first *in vivo* use of calcium orthophosphates was performed in 1920; that time the researchers implanted tricalcium phosphate (TCP) into animals to test its efficacy as a bone substitute [1]. In the following years, some other calcium orthophosphates were tested on animals to investigate their effect on the healing of nonunions [2]. However, it was 1951, when for the first time hydroxyapatite (HA) was implanted in rats and guinea pigs [3]. Those attempts might be characterized as initial medical trials with the first generation of bone substituting biomaterials. However, it was already the 1970-s, when other calcium orthophosphates were synthesized, characterized, investigated and tried in medicine [4-10]. The list of known calcium orthophosphates, including their standard abbreviations and the major properties, is shown in Table 1 [11].

The possibility to obtain a monolithic calcium orthophosphate ceramics at ambient or body temperature via a cementation reaction was put forward by the scientists at the American Dental Association LeGeros *et al.* [12] and Brown and Chow [13-16] in the early 1980-s. However, there is an opinion [17] that the self-setting calcium orthophosphate cements for orthopedic and dental restorative applications have first been described in the early 1970-s by Driskell *et al.* [18]. More to the point, some researchers worked with similar reactions even earlier. For example, Kingery looked at formulations based on CaO and H_3PO_4 in 1950 [19]. Currently this type of materials is known as *calcium phosphate cements* (commonly referred to as CPC), and, due to their suitability for repair, augmentation and regeneration of bones, they might be named as calcium phosphate *bone* cements (occasionally referred to as CPBC) [20]. In order to stress the fact, that these cements consist either entirely or essentially of calcium *orthophosphates*, this review is limited to consideration of calcium orthophosphate cements only. Due to a good bioresorbability, calcium orthophosphate cements belong to the second generation of bone substituting biomaterials [21]. These cements are blends of amorphous and/or crystalline calcium orthophosphate powder(s) with an aqueous solution, which might be distilled water, phosphate-buffered saline (PBS), ~ 0.25 M aqueous solution of sodium orthophosphate, orthophosphoric acid, ~ 0.5 M aqueous solution of citric acid [22] or even revised simulated body fluid (rSBF) [23].

After the powder(s) and the solution are mixed together, a viscous and moldable paste is formed that sets to a firm mass within a few minutes. When the paste becomes sufficiently stiff, it can be placed into a defect as a substitute for the damaged part of bone, where it hardens *in situ* within the operating theatre. The proportion of solid to liquid or the powder-to-liquid (P/L) ratio is a very important characteristic because it determines both bioresorbability and rheological properties. As the paste is set and hardened at room or body temperature, direct application in healing of bone defects became a new and innovative treatment modality by the end of the XX-th century. Moreover, calcium orthophosphate cements can be injected directly into the fractures and bone defects, where they intimately adapt to the bone cavity regardless its shape. More to the point, they were found to promote development of osteoconductive pathways, possess sufficient compressive strengths, be noncytotoxic, create chemical bonds to the host bones, restore contour and have both the chemical composition and X-ray diffraction patterns similar to those of bone [24]. Finally but

yet importantly, they are osteotransductive, *i.e.*, after implantation, calcium orthophosphate cements are replaced by a new bone tissue [25-27].

Table 1. Existing calcium orthophosphates and their major properties [11].

Ca/P ionic ratio	Compound	Chemical formula	Solubility at 25 °C, – log(K _s)	Solubility at 25 °C, g/L	pH stability range in aqueous solutions at 25°C
0.5	Monocalcium phosphate monohydrate (MCPM)	Ca(H ₂ PO ₄) ₂ ·H ₂ O	1.14	~ 18	0.0 – 2.0
0.5	Monocalcium phosphate anhydrous (MCPA)	Ca(H ₂ PO ₄) ₂	1.14	~ 17	^[c]
1.0	Dicalcium phosphate dihydrate (DCPD), mineral brushite	CaHPO ₄ ·2H ₂ O	6.59	~ 0.088	2.0 – 6.0
1.0	Dicalcium phosphate anhydrous (DCPA), mineral monetite	CaHPO ₄	6.90	~ 0.048	^[c]
1.33	Octacalcium phosphate (OCP)	Ca ₈ (HPO ₄) ₂ (PO ₄) ₄ ·5H ₂ O	96.6	~ 0.0081	5.5 – 7.0
1.5	α-Tricalcium phosphate (α-TCP)	α-Ca ₃ (PO ₄) ₂	25.5	~ 0.0025	^[a]
1.5	β-Tricalcium phosphate (β-TCP)	β-Ca ₃ (PO ₄) ₂	28.9	~ 0.0005	^[a]
1.2 – 2.2	Amorphous calcium phosphate (ACP)	Ca _x H _y (PO ₄) _z ·nH ₂ O, n = 3 – 4.5; 15 – 20% H ₂ O	^[b]	^[b]	~ 5 – 12 ^[d]
1.5 – 1.67	Calcium-deficient hydroxyapatite (CDHA) ^[e]	Ca _{10-x} (HPO ₄) _x (PO ₄) _{6-x} (OH) _{2-x} ^[f] (0 < x < 1)	~ 85.1	~ 0.0094	6.5 – 9.5
1.67	Hydroxyapatite (HA)	Ca ₁₀ (PO ₄) ₆ (OH) ₂	116.8	~ 0.0003	9.5 – 12
1.67	Fluorapatite (FA)	Ca ₁₀ (PO ₄) ₆ F ₂	120.0	~ 0.0002	7 – 12
2.0	Tetracalcium phosphate (TTCP), mineral hilgenstockite	Ca ₄ (PO ₄) ₂ O	38 – 44	~ 0.0007	^[a]

^[a] These compounds cannot be precipitated from aqueous solutions.

^[b] Cannot be measured precisely. However, the following values were found: 25.7 ± 0.1 (pH = 7.40), 29.9 ± 0.1 (pH = 6.00), 32.7 ± 0.1 (pH = 5.28).

^[c] Stable at temperatures above 100 °C.

^[d] Always metastable.

^[e] Occasionally, CDHA is named as precipitated HA.

^[f] In the case x = 1 (the boundary condition with Ca/P = 1.5), the chemical formula of CDHA looks as follows: Ca₉(HPO₄)(PO₄)₅(OH).

Table 2. Some calcium orthophosphate cement formulations having the 510(k) clearance from the FDA [20, 111, 172]. The technical data on these cements might be found in literature [21].

Product*	Manufacturer	Applications*
BoneSource ^{TM**}	Striker Howmedica Osteonics (Rutherford, NJ)	Craniofacial
α -Bone Substitute Material (α -BSM ^{®***})	Etex Corporation (Cambridge, MA)	Filling of bone defects and voids, dental, craniofacial
Skeletal Repair Systems (SRS [®])	Norian Corporation (Cupertino, CA)	Skeletal distal radius fractures, craniofacial

* In Europe, other applications may apply, and the materials may be sold with a different commercial name.

** BoneSourceTM is the original formulation of calcium orthophosphate cement developed by Brown and Chow.

*** In Europe, it is distributed by Biomet Merck (Zwijndrecht, The Netherlands) as Biobon[®] [111], while in North America it is marketed by Walter Lorenz Surgical (Jacksonville, FL) as Embarc[®] [21].

The aim of biomimetic bone cements is to disturb bone functions and properties as little as possible and, until a new bone has been grown, to behave temporary in a manner similar to that of bone. From a biological point of view, this term defines cements that can reproduce the composition, structure, morphology and crystallinity of bone crystals [28, 29]. Therefore, the discovery of self-setting calcium orthophosphate cements was a significant step forward in the field of bioceramics for bone regeneration, since it established good prospects for minimally invasive surgical techniques that were less aggressive than the classical surgical methods [30]. The cements provide the surgeons with a unique ability of manufacturing, shaping and implanting the bioactive bone substitute material on a patient-specific base in real time in the surgery room. Implanted bone tissues also take benefits from initial setting characteristics of the cements that give, in an acceptable clinical time, a suitable mechanical strength for a shorter tissue functional recovery. The major advantages of the cements include a fast setting time, excellent moldability, outstanding biocompatibility and easy manipulation; therefore, the cements are more versatile in handling characteristics than prefabricated calcium orthophosphate granules or blocks. Besides, like any other bioceramics, calcium orthophosphate cements provide the opportunity for bone grafting using alloplastic materials, which are unlimited in quantity and provide no risk of infectious diseases [31-33].

From the point of view that calcium orthophosphate cements are intended for using as biomaterials for parenteral application, for their chemical composition one might employ all ionic compounds of oligoelements occurring naturally in a human body. The list of possible additives includes (but is not limited to) the following cations: Na⁺, K⁺, Mg²⁺, Ca²⁺, Sr²⁺, H⁺ and anions: PO₄³⁻, HPO₄²⁻, H₂PO₄⁻, P₂O₇⁴⁻, CO₃²⁻, HCO₃⁻, SO₄²⁻, HSO₄⁻, Cl⁻, OH⁻, F⁻, SiO₄⁴⁻ [25]. Therefore, mixed-type cements consisting of calcium orthophosphates and other calcium salts (*e.g.*, gypsum [34, 38, 39], calcium sulfate hemihydrate [40], calcium pyrophosphate [41-43], calcium polyphosphates [44], calcium carbonate [29, 45-47], calcium oxide [48-53], calcium hydroxide [54-56], calcium aluminate [57, 58], calcium silicate [59-62], *etc.*), strontium orthophosphate [63, 64], magnesium orthophosphate [65-67], barium sulfate [68], as well as cements made of various ion substituted calcium orthophosphates

(e.g., $\text{Ca}_2\text{KNa}(\text{PO}_4)_2$, NaCaPO_4 , $\text{Na}_3\text{Ca}_6(\text{PO}_4)_5$, magnesium substituted CDHA, strontium substituted CDHA, etc.) [69-77] are available. Moreover, calcium orthophosphate cements might be prepared in the reaction-setting mixture of $\text{Ca}(\text{OH})_2 - \text{KH}_2\text{PO}_4$ system [78], as well as by treatment of calcium carbonates with orthophosphate solutions [79]. Calcium orthophosphate cements possessing magnetic properties due to incorporation of iron oxides have been developed as well [80, 81]. However, with a few important exceptions, such formulations have not been considered in this review.

The purpose of this article is to review the chemistry, physical and mechanical properties of calcium orthophosphate cements with the specific reference to their biomedical applications in dentistry and surgery.

2. CALCIUM ORTHOPHOSPHATE CEMENTS

According to Wikipedia, the free encyclopedia: “In the most general sense of the word, *cement* is a binder, a substance that sets and hardens independently and can bind other materials together. The name “cement” goes back to the Romans who used the term “*opus caementitium*” to describe masonry, which resembled concrete and was made from crushed rock with burnt lime as binder. The volcanic ash and pulverized brick additives, which were added to the burnt lime to obtain a hydraulic binder, were later referred to as *cementum*, *cimentum*, *cäment* and *cement*” [82]. Thus, *calcium orthophosphate cement* appears to be a generic term to describe chemical formulations in the ternary system $\text{Ca}(\text{OH})_2 - \text{H}_3\text{PO}_4 - \text{H}_2\text{O}$ which can experience a transformation from a liquid or pasty state to a solid state and in which the end-product of the chemical reactions is a calcium orthophosphate.

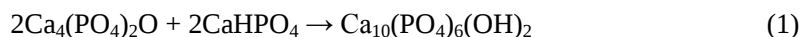
The first calcium orthophosphate cement formulation consists of the equimolar mixture of TTCP and dicalcium phosphate (DCPA or DCPD) [83] which is mixed with water at a P/L ratio of 4:1; the paste hardened in about 30 min and formed CDHA [14, 15]. This highly viscous, non-injectable paste can be molded and is therefore used mainly as a contouring material in craniofacial surgery. In 1990-s, it was established that there were about 15 different binary combinations of calcium orthophosphates, which gave pastes upon mixing with water or aqueous solutions, so that the pastes set at room or body temperature into a solid cement. The list of these combinations is available in literature [86-88]. From these basic systems, secondary formulations could be derived containing additional or even non-reactive compounds but still setting like cements [25, 50, 86, 89-102]. According to the classical solubility data, depending upon the pH value of a cement paste, after setting all calcium orthophosphate cements can only form two major end-products: a precipitated poorly crystalline HA or CDHA [103] at $\text{pH} > 4.2$ and DCPD (also called “brushite” [104]) at $\text{pH} < 4.2$ [105]. However, in the real cement formulations this pH-border is shifted to a higher value of pH. Namely, DCPD might be formed at pH up to ~ 6 , while CDHA normally is not formed at pH below 6.5 – 7 (Table 1). The results of the only study on an ACP cement [100] demonstrated that this end-product was rapidly converted into CDHA. Besides, there is one paper devoted to an OCP cement [106]; however, contrary to reports of early 1990-s, none of the calcium orthophosphate cements synthesized afterwards was of OCP or ACP types. Therefore, all existing formulations of calcium orthophosphate cements have been divided into two major groups: apatite cements and brushite cements [107]. The final hardened

product of the cements is of the paramount importance because it determines the solubility and, therefore, *in vivo* bioresorbability. Since the chemical composition of mammalian bones is similar to an ion-substituted CDHA, apatite cements have been more extensively investigated. However, many research papers on brushite cements have been published as well.

All calcium orthophosphate cements are made of an aqueous solution and fine powders of one or several calcium orthophosphate(s). Here, dissolution of the initial calcium orthophosphates (quickly or slowly depending on the chemical composition and solution pH) and mass transport appear to be the primary functions of an aqueous environment, in which the dissolved reactants form a supersaturated (very far away from the equilibrium) microenvironment with regard to precipitation of the final products [109, 110]. The relative stability and solubility of various calcium orthophosphates is the major driving force for the setting reactions that occur in these cements. Therefore, mixing of a dry powder with an aqueous solution induces various chemical transformations, where crystals of the initial calcium orthophosphate(s) rapidly dissolve(s) and precipitate(s) into crystals of CDHA or DCPD with possible formation of intermediate precursor phases (*e.g.*, ACP and OCP). During precipitation, the newly formed crystals grow and form a web of intermingling microneedles or microplatelets of the final products, thus provide a mechanical rigidity to the hardened cements. In other words, entanglement of the newly formed crystals is the major reason of setting. For the majority of apatite cements, water is not a reactant in the setting reaction. Therefore, the quantity of water, actually needed for setting of apatite cements, is very small [21, 109, 111]. However, for brushite cements, water always participates in the chemical transformations because it is necessary for DCPD formation. Due to this reason, brushite cements are always hydraulic, while usually this term is not associated with apatite cements.

Setting of calcium orthophosphate cements is a continuous process that always starts with dissolution of the initial compounds in an aqueous system. This process supplies ions of calcium and orthophosphate into the solution, where they chemically interact and precipitate in the form of either the end-products or precursor phases, which causes the cement setting [112-114]. This was confirmed by Ishikawa and Asaoka, who showed that when TTCP and DCPA powders were mixed in double-distilled water, both powders were dissolved. The dissolved calcium and orthophosphate ions in the solution were then precipitated in the form of CDHA on the surface of the powders [115]. The precipitate can be either a gel or a conglomerate of crystals. Therefore, the hardening mechanism is either a sol-gel transition of ACP [100] or entanglement of the precipitated crystals of other calcium orthophosphates [25]. For example, for the classical Brown-Chow cement formulation, after the initial setting, petal or needle-like crystals enlarge epitaxially and are responsible for the adherence and interlocking of the crystalline grains, which result in hardening. After ~ 2 hours, the newly formed crystals become rod-like, resulting from higher crystallinity with the observation of more material at the inter-particle spaces. During this period, the cement setting reaction proceeded at a near-constant rate, suggesting that the reaction rate was limited by factors that are unrelated to the amounts of the starting materials and the reaction products present in the system. Such factors could be related to the surface area of DCPA or TTCP or to the diffusion distances over which the calcium and orthophosphate ions migrate in order to form CDHA [116-118]. At ~ 24 hours, the crystals are completely formed, being very compacted in some areas of high density and well separated in areas with more porosity [93, 98, 99].

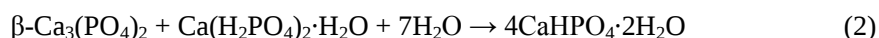
The chemical reactions that take place during the setting of calcium orthophosphate cements depend on their chemical composition. However, it can be stated that only two major chemical types of the setting reaction are possible. The first type occurs according to the classical rules of the acid-base interaction, *i.e.* a relatively acidic calcium orthophosphate reacts with a relatively basic one to produce a relatively neutral compound. The first cement by Brown and Chow is a typical example of this type because TTCP (basic) reacts with DCPA (slightly acidic) in an aqueous suspension to form a precipitated poorly crystalline HA (slightly basic) [14, 15]:



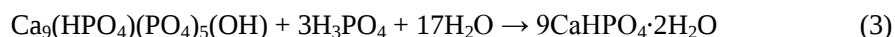
Earlier, it was believed that DCPA and TTCP reacted upon mixing with water to form the stoichiometric HA [13-16]. However, further investigations have shown that only the first nuclei consist of a nearly stoichiometric HA, whereas further growth of these nuclei occurs in the form of CDHA [119]. Besides, there is a study demonstrating that the initially formed HA further interacts with remaining DCPD to form CDHA [120].

Formation of HA according to equation (1) releases neither acidic nor basic byproducts. Thus, the liquid phase of the cement remains at a near constant pH of ~ 7.5 for the TTCP + DCPD and ~ 8.0 for the TTCP + DCPA formulations, respectively [116-118]. Various deviations from the stoichiometry of chemical equation (1) were studied in details and various apatitic calcium orthophosphates with Ca/P ionic ratio within 1.5 – 1.67 were found as the end-product [121]. The effect of mixing ratio and pH on the reaction between TTCP and DCPA is well described elsewhere [122]. Furthermore, the influence of Ca/P ionic ratio of TTCP on the properties of the TTCP + DCPD cement was studied as well [123].

A blend proposed by Lemai *et al.* [124, 125] is another example of the acid-base interaction where β -TCP (almost neutral) reacts with MCPM (acidic) to form DCPD (slightly acidic):



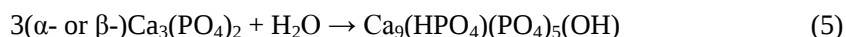
In chemical equation (2) MCPM might easily be substituted by orthophosphoric acid [126-129] or MCPA, while β -TCP might be replaced by either α -TCP [130, 131] or CDHA [132, 133]. For example:



Furthermore, cement formulations based on mixtures of ACP + α -TCP [134], ACP + DCPD [135, 136], DCPA + α -TCP [131], OCP + TTCP [137] and partially crystallized calcium orthophosphate + DCPA [138] as the initial reagents, are also available.

The second type of the setting reaction might be defined as hydrolysis of a metastable calcium orthophosphate in aqueous media. As the result, both the initial and final compounds have the same Ca/P ionic ratio. Due to the fact, that only one calcium orthophosphate is used; the solid part of such formulations might be called as a single-phase (or single-component) cement powder [139]. Cements made of ACP + an aqueous solution [140, 141], α -TCP + an aqueous solution [142-148], β -TCP + an aqueous solution [146, 149], nanocrystalline TTCP

+ an aqueous solution [150, 151] or γ -radiated TTCP + an aqueous solution [152-154] are the typical examples; all of them re-crystallize to CDHA upon contact with water:



The experimental details on TTCP hydrolysis under a near-constant composition condition might be found elsewhere [155]. The details on α -TCP hydrolysis are also available. The results indicated that setting of α -TCP was initially controlled by surface dissolution; therefore, it depended on the surface area of the reactants [156-159]. Hydrolysis of DCPD to CDHA was studied as well [160]. Furthermore, addition of ~ 2 wt. % of a precipitated poorly crystalline HA as a seed to α -TCP powder phase might be useful to accelerate the kinetics of reaction (5) [161].

Further, there is a single-phase cement powder consisting of K- and Na- containing CDHA (with the Ca/P ionic ratio of 1.64 ± 0.02) that sets and hardens after mixing with an aqueous solution of sodium citrate and sodium orthophosphate [17]. After setting, this formulation gives rise to formation of a weak cement (the compressive strength of 15 ± 3 MPa) consisting of the ion-substituted CDHA again (presumably, with a different Ca/P ionic ratio), mimicking the bone mineral. Unfortunately, neither the setting reaction nor the setting mechanism of this cement has been disclosed in literature [17]. What's more, a self-setting cement might be prepared from the thermal decomposition product of HA [162].

The hydration process of calcium orthophosphate cements is slightly exothermic (which is beneficial for biomedical applications) and undergoes five periods: initiating period, induction period, accelerating period, decelerating period and terminating period [163]. For the classical Brown-Chow cement formulation, the activation energy of the hydration reaction is 176 kJ/mol [164]. The rate of heat liberation during the solidification of calcium orthophosphate cements is low. The results of adiabatic experiments showed that the temperature rise arrived at the highest value of 37 °C 3 h later, which would cause no harm to surrounding tissues [163]. The results show that the hardening process of this cement is initially controlled by the dissolution of the reactants in a 4 h period and subsequently by diffusion through the product layer of CDHA around the grains [99]. In general, setting of calcium orthophosphate cements occurs mostly within the initial ~ 6 hours, yielding an ~ 80 % conversion to the final products. The volume of the cements stays almost constant during setting. However, after hardening, calcium orthophosphate cements always form brittle ceramics with the tensile strength of 5 to 20 times lower than the compression strength [165, 166]. Since this material is weak under tensile forces, these cements can only be used either in combination with metal implants or in non-load bearing (*e.g.*, craniofacial) applications [111, 167-169]. This is confirmed by the mechanical characterization of a bone defect model filled with ceramic cements [170].

To conclude this part, one must stress, that chemical equations (1) – (6) of the cement setting are valid for the *in vitro* conditions only. There are evidences that samples of calcium orthophosphate cement retrieved 12 h after hardening *in vivo* already contained carbonateapatite, even though the initial cement mixture did not contain carbonate as one of

the solid components [171]. The mass fraction of carbonate in the 12 h samples was about 1 %. The results suggest that under the *in vivo* conditions, carbonate is readily available and this allows formation of carbonateapatite in favor of carbonate-free CDHA [171].

The United States Food and Drug Administration (FDA) has approved several cement formulations for clinical use [21, 172]. Some examples are given in Table 2. The same formulations have also received a Conformite Europeene (CE) mark for certain maxillofacial indications and for use as a bone-void filler in the specific non-load-bearing orthopedic indications [111]. The major properties of these formulations are available in literature [21]. The list of other commercially available injectable bone cements with their chemical composition (when obtainable) might be found elsewhere [30, 118, 173, 174], while the various types of bone cements and fillers are listed in another review [168]. Besides, many more cement formulations are in experimental stages. A general appearance of two randomly chosen commercial calcium orthophosphate cements is shown in Figure 1.

3. TWO MAJOR TYPES OF CALCIUM ORTHOPHOSPHATE CEMENTS

3.1. Apatite Cements

Typically, apatite cement formulations have a precipitated poorly crystalline HA and/or CDHA as the end-product of the setting reaction (see chemical equations (1), (4) – (6)), although traces of the unreacted starting materials can be present [93]. Due to the initial presence of carbonates, such apatite cements as Norian SRS[®] and Biocement D[®] form a non-stoichiometric carbonatapatite or dahllite ($\text{Ca}_{8.8}(\text{HPO}_4)_{0.7}(\text{PO}_4)_{4.5}(\text{CO}_3)_{0.7}(\text{OH})_{1.3}$) as the end-product [45, 175]. As both CDHA and carbonateapatite are formed in an aqueous environment and have a low crystallinity, they appear to be similar to biological apatite of bones and teeth. These properties are believed to be responsible for the excellent *in vivo* resorption characteristics. Conventional apatite cements contain TCP and/or TTCP phases in their powder components [30], while a single component cement powder consisting of K- and Na- containing CDHA is also available [17]. The reactivity of TCP-based apatite cements varies as a function of TCP crystal phase, crystallinity and particle size [176, 177]. Generally, a higher reactivity is observed with a thermodynamically less stable phase (from β -TCP to α -TCP and ACP) and with a smaller particle size [146]. Nominally, it might be stated that formation of apatites through a cementation reaction is a sort of a biomimetic process because it occurs in physiological environment and at body temperature [33]; however, both the crystallization kinetics and a driving force are very far away from the biomimeticity. A unique feature of the hardened apatite cements is that the force linking the newly formed crystals (of both CDHA and carbonatapatite) is weak; therefore, the crystals can be easily detached from the cement bulk, especially after dissolution has partly occurred. When this happens, osteoclasts and other cells can easily ingest the apatite crystals [178].

Immediately after implantation, any cement becomes exposed to blood and other tissue fluids that delays the setting time. Intrinsic setting time for apatite cements has been extensively studied and it appeared to be rather long. For example, for the original formulation by Brown and Chow it ranged from 15 to 22 min [14, 15]. This may result in procedural complications. To remedy this, the amount of liquid might be reduced to a

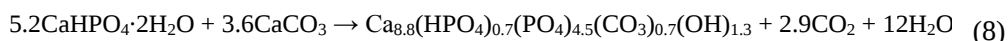
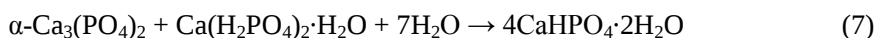
possible minimum. Therefore, all apatite cements are viscous and easily moldable pastes but tend to be difficult to inject. Besides playing with the P/L ratio, the setting time can also be reduced by using additives to the liquid phase (which is distilled water in the Brown-Chow formulation [14, 15]). The list of additives includes phosphoric acid, MCPM and other soluble orthophosphates. These additives promote dissolution of the solids by lowering the solution pH. In such cases, a setting time in the range of 10 – 15 minutes can be obtained [140, 142-148, 179]. The influence of soluble orthophosphates (*e.g.*, Na_2HPO_4 or NaH_2PO_4) on the setting time of apatite cements is explained by the fact that dissolution of DCPA and formation of CDHA during setting occur in a linear fashion, thus avoiding early formation of CDHA. This is important because too early formation of CDHA might engulf un-reacted DCPA, which slows down DCPA dissolution and thus the setting kinetics becomes slower, while the presence of sodium orthophosphates prevents DCPA particles from being isolated [180]. Particle size [161, 181, 182], temperature of the liquid phase and initial presence of HA as a seed in the solid phase are other factors that influence the setting time [14, 15, 33, 176, 177]; however, *in vitro* studies demonstrated that these parameters did not affect significantly [93]. On the other hand, a reduction in particle size was found to result in a significant decrease in both initial and final setting times [161, 181, 182], an acceleration of the hardening rate [161] and hydration kinetics of the hardening cement [182]. Besides, the crystallite sizes of the final product can be strongly reduced by increasing the specific surface of the starting powder, which allows developing calcium orthophosphate cements with tailored structures at the micro and nanoscale levels [161]. Unfortunately, an unclear correlation was found between the particle dimensions of the initial calcium orthophosphates and mechanical properties of the hardened cements: namely, a significant increase in compressive strength and storage modulus was reported for some formulations [181, 182] but a minor effect on compressive strength was discovered for other ones [161]. This inconsistency is not surprising because the manufacturing method used to produce test samples varies from one author to the other. Therefore, the only remaining fact is that calcium orthophosphate cements are brittle and hence worthless for load-bearing applications [167, 168].



Figure 1. A presentation of two randomly chosen commercial calcium orthophosphate cements.

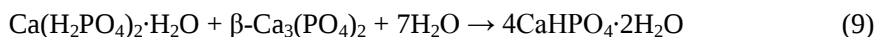
Setting process of the most types of apatite cements occurs according to just one chemical reaction (see chemical equations (1), (4) – (6)) and at near the physiological pH. The latter may additionally contribute to the high biocompatibility observed for these materials [116-118]. For the classical formulation by Brown and Chow, the transmission electron microscopy results suggested the process for early-stage apatite formation as follows: when TTCP and DCPA powders were mixed in an orthophosphate-containing solution, TTCP powder was quickly dissolved due to its higher solubility in acidic media. Then the dissolved ions of calcium and orthophosphate, along with ions already existing in the solution, were precipitated predominantly onto the surface of DCPA particles. Few apatite crystals were observed on the surface of TTCP powder. At a later stage of the reaction, an extensive growth of apatite crystals or whiskers effectively linked DCPA particles together and bridged the larger TTCP particles causing the cement setting [183].

However, Norian SRS[®] and Cementek[®] were found to set according to two chemical reactions: precipitation of DCPD, followed by precipitation of either CDHA or carbonatapatite:

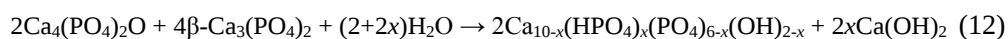
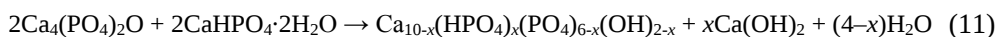


The initial chemical reaction (7) was very fast and provoked DCPD formation and setting of the cement pastes within seconds. The second step was slower: DCPD reacted completely within several hours with remaining $\alpha\text{-Ca}_3(\text{PO}_4)_2$ and CaCO_3 forming carbonatapatite according to equation (8). The latter step caused the cement hardening. A similar two-step hardening mechanism was established for a cement consisting of MCPM and CaO: in the first step, during the mixing time, MCPM reacted with CaO immediately to give DCPD, which, in the second step, reacted more slowly with the remaining CaO to give CDHA [50].

The aforementioned setting mechanism of an apatite cement was investigated in details for a three component mixture of TTCP, $\beta\text{-TCP}$ and MCPM dry powders in convenient proportions and with the overall atomic Ca/P ratio equal to 1.67. Two liquid phases in a raw were used to damp the cement powder, initially it was water + ethanol (ethanol was added to slow down the hardening) and afterwards orthophosphoric acid and sodium glycerophosphate were added to water to prepare a reactive liquid [109]. At the very beginning, DCPD was found to form according to two chemical reactions:



The formation reactions of DCPD were fast and corresponded to the setting stage. Afterwards, TTCP reacted with the previously formed DCPD and with $\beta\text{-TCP}$ to give CDHA according to the reactions:



The formation reactions of the CDHA phase were quite slow and corresponded to the hardening stage. Although OCP was not detected in that study, its formation as an intermediate phase was postulated for this cement [109]. A similar suggestion on the intermediate formation of OCP was made for the setting mechanism of Brown-Chow classical cement formulation [88, 93]; however, a reliable evidence for its presence is still lacking [143, 184]. In both cases, OCP was suggested to appear as an intermediate because it was a faster forming phase than CDHA. This hypothesis is based upon the classical studies performed by Prof. W. E. Brown *et al.* about the precursor phase formation during chemical crystallization of apatites in aqueous solutions [185-187].

Solubility of the hardened apatite cements in aqueous solutions is expected to be rather similar to that of bone mineral. This means that they are relatively insoluble at neutral pH and increasingly soluble as pH drops down; this is an important characteristic of normal bone mineral that facilitates controlled dissolution by osteoclasts [175].

To conclude this part, one should mention, that in 2000 the US bone substitute market for Norian SRS[®] accounted for ~ 15 % of the total sales, followed by BoneSource[™] at ~ 13 %, and α -BSM[®] at ~ 8.5 % [111].

3.2. Brushite Cements

As indicated by its name, DCPD is the major end-product of the setting reaction of brushite cements (chemical equations (2) and (3)). Mirtchi and Lemaitre [124] and independently Bajpai *et al.* [125] introduced this type of the cements in 1987. Up to now, several formulations have been already proposed, *e.g.*, β -TCP + MCPM [124, 126], β -TCP + H_3PO_4 [125, 127, 128] and TTCP + MCPM + CaO [188]. All brushite cements are set by the acid-base interaction only. As DCPD can only precipitate at the solution pH < 6, the paste of brushite cement is acidic during setting [127, 189]. For example, during setting of a β -TCP + MCPM cement, the cement pH varies from very acidic pH values of ~ 2.5, to almost neutral pH values of ~ 6.0 [127]. Replacing MCPM by orthophosphoric acid renders the cement paste very acidic for the initial ~ 30 s but then the pH profile follows that obtained with MCPM. It is important to notice, that β -TCP + H_3PO_4 formulations have several advantages over β -TCP + MCPM formulations, namely: (i) easier and faster preparation, (ii) a better control of the chemical composition and reactivity, (iii) improved physico-chemical properties, such as longer setting times and larger tensile strengths due to a higher homogeneity. However, the use of orthophosphoric acid might impair the biocompatibility of the cement formulation, due to low pH values during setting [127]. If a cement paste contains an excess of a basic phase, the equilibrium pH will be given by the intersection of the solubility isotherms of the basic phase with that of DCPD. For example, the equilibrium pH values of β -TCP + MCPM, HA + MCPM and TTCP + MCPM mixtures are 5.9, 4.2 and 7.6, respectively [167, 168].

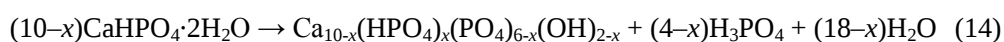
As the solubility of calcium orthophosphates decreases with increasing of their basicity (Table 1), the setting time of brushite cements much depends on the solubility of a basic phase: the higher its solubility, the faster the setting time. Therefore, the setting time of the cements made of MCPM + a basic calcium orthophosphate increases in the order: HA > β -TCP > α -TCP [167, 168]. For example, HA + MCPM mixtures have a setting time of several minutes, β -TCP + MCPM mixtures – of 30 to 60 seconds and α -TCP + MCPM mixtures – of a few seconds [124, 125]. Despite this initial high reactivity, the hardening reaction of

brushite cements typically lasts one day until completion [176, 177]. Additives that inhibit the crystal growth of DCPD have successfully been used to increase the setting time of β -TCP + MCPM mixtures [190]. In contrast to apatite cements, brushite cements can be initially liquid and still set within a short period of time [167, 168].

Brushite is remarkably biocompatible and bioresorbable [189]. Due to both a better solubility of DCPD if compared to that of CDHA (Table 1) and metastability of DCPD under physiological conditions [191], brushite cements are faster degradable than apatite cements [192-194]. They are quickly resorbed *in vivo* and suffered from a rapid decrease in strength (although the mechanical properties of the healing bone increase as bone ingrowth occurs [31]). Short setting times, low mechanical strength and limited injectability seem to prevent brushite cements from a broader clinical application. However, the major reason why brushite cements are not more widespread is probably not related to the mechanical issues but just to a later arrival on the market. Use of sodium citrate or citric acid as setting retardants is an option to get more workable and less viscous pastes of brushite cements [22, 195-198]. Similar effect might be achieved by addition of chondroitin 4-sulfate [199] and glycolic acid [200]. For the cement formulations with orthophosphoric acid as the initial reactant (see chemical equation (3)), acid deficient formulations were also found to improve the workability. In this case, the setting reaction might be described by the following chemical equation [198]:



Although, several studies revealed that too much of DCPD in a given volume was not detrimental to the biological properties of brushite cements [31, 175, 188], occasionally, when large quantities of brushite cements were used, a certain degree of tissue inflammation during the first weeks of *in vivo* implantation were reported [194, 198, 201]. Further investigations indicated that the inflammatory could be due to a partial transformation of DCPD into CDHA with release of orthophosphoric acid [202]:



Transformation of DCPD into CDHA occurs via two successive processes: dissolution and precipitation [203] and can be retarded by adding magnesium ions to the cement paste, thus reducing the possibility of inflammation [167, 168]. The aforementioned case of acid deficient formulations of brushite cements (chemical equation (13)) is an alternative, because it reduces the amount of unreacted acid in the cement [198] with an option to consume liberating in chemical equation (14) orthophosphoric acid by the excess of β -TCP. Implantation of previously set brushite cement might be the third option, because a solid material was found to be better tolerated than paste implants. Besides, more bone was formed at the solid implant contact and the solid material degraded not so rapidly [204]. For brushite cements, a linear degradation rate of 0.25 mm/week was reported [205]. This rapid degradation rate might lead to formation of an immature bone. Adding β -TCP granules to the cement paste could solve this problem because β -TCP granules might act as bone anchors and encourage formation of a mature bone [205, 206].

4. VARIOUS PROPERTIES OF CALCIUM ORTHOPHOSPHATE CEMENTS

Generally, calcium orthophosphate cements must set slowly enough to provide sufficient time to a surgeon to perform implantation but fast enough to prevent delaying the operation. Ideally, good mechanical properties should be reached within minutes after initial setting. Two main experimental approaches are used to study the cement setting process: a batch approach and a continuous approach. In the batch approach, the setting reaction is stopped at various times and the resulting samples are analyzed to determine *e.g.*, the composition and compressive strength of the samples [176, 177]. There are currently two standardized methods to apply this approach, namely, Gillmore needles method (ASTM C266-89) [207] and Vicat needle method (ASTM C191-92) [208]. The idea of both methods is to examine visually the surface of cement samples to decide whether the cement has already set, *i.e.* if no mark can be seen on the surface after indentation. Besides, the setting process might be monitored in real time by non-destructive methods (the continuous approach), *e.g.*, pulse-echo ultrasound technique [209, 210], isothermal differential scanning calorimetry [145, 146, 211-216] and alternating current (AC) impedance spectroscopy [217]. For example, recent calorimetry measurements suggested that in equation (2) the endothermic MCPM dissolution and the highly exothermic β -TCP dissolution occurred simultaneously, followed by the exothermic crystallization of DCPD [215]. Moreover, acid-base reactions (1) – (3) can be and have been analyzed by measuring the pH evolution of a diluted cement paste [176]. Finally yet importantly, methods of Fourier-transform infrared spectroscopy [216, 218], X-ray diffraction [43, 130, 219] and energy dispersive X-ray diffraction [220] might be applied as well. The latter techniques proved to be powerful even though they have limitations such as the time required for each measurement (250 s for an X-ray diffraction scan is a problem for fast setting reactions); besides the analysis is located at the surface of the sample where evaporation and thermal effects can modify the reaction rate of the surface compared to that of the bulk. Furthermore, the continuous approach is an indirect one, which markedly complicates an interpretation of the collected data, particularly in complex cement formulations [176].

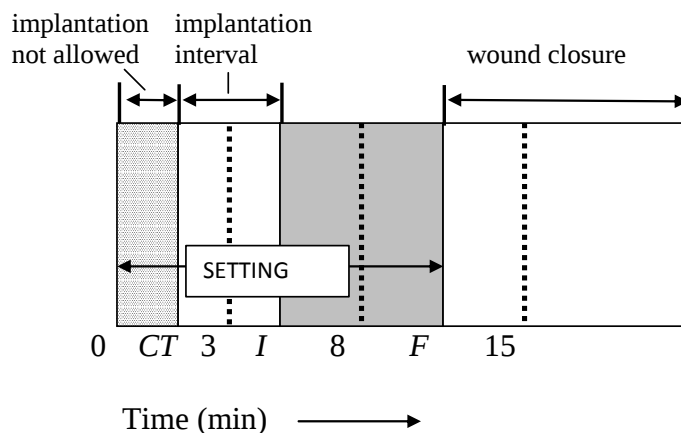


Figure 2. A diagram of the setting parameters relevant for a calcium orthophosphate cement: CT – cohesion time; I – initial setting time; F – final setting time. Adapted from Ref. [25] with permission.

A way to assess the rate of a cement hardening is to measure its setting time, which means the time required to reach a certain compressive strength, generally close to 1 MPa. The most straightforward approach is to prepare cement samples with a well-controlled geometry (*e.g.*, cylinders), incubating these samples for various times in the right environment (temperature, humidity) and assessing the composition and mechanical properties of the samples as a function of time [176]. One should stress, that setting time for calcium orthophosphate cements often corresponds to an earlier stage in the overall setting reaction, typically 5 – 15 % of the overall reaction, while the end of the cement setting is typically reached after several days [93, 143]. Gillmore needles have been used with success to measure the initial (*I*) and final (*F*) setting times of calcium orthophosphate cements [86]. A light and thick needle is used to measure the initial setting time *I*, while a heavy and thin needle for the final setting time *F* [108]. The clinical meaning is that the cement paste should be implanted before time *I* and that the wound can be closed after time *F* (Figure 2).

The cement should not be deformed between times *I* and *F* because in that stage of the setting process any deformation could induce cracks [25]. The following handling requirements (in minutes) have been formulated for calcium orthophosphate cements, as a result [108, 221]:

$$3 \leq I < 8$$

$$I - CT \geq 1$$

$$F \leq 15$$

These parameters are represented schematically in Figure 2. The second requirement means, that the cohesion time (*CT*) must be at least 1 min before *I*, so that a clinician has at least 1 min to apply and to mold the material. *CT* is the time from which a cement no longer disintegrates when immersed in Ringer's solution [108]. As the mixing in a mortar is about 1 min, the shortest *CT* that can be allowed is about 2 min, so that a clinician has at least 1 min to collect the paste from the mortar and put it on the pallet knife or in the syringe with which it is to be transferred to the wound after *CT* and before *I* [108]. For dental applications, time *I* must be close to 3 min, whereas for orthopedic applications it must be close to 8 min. However, in no case it will be tolerable for the clinicians if time *F* becomes greater than 15 min [25, 108].

In the clinical situation, calcium orthophosphate cements can be either applied by the fingertips of a surgeon or injected from a syringe to the defect area of a bone. The first type of clinical application requires formulation of a high-viscosity cement paste, which can be applied manually as dough, while the second type of clinical application requires formulation of a low-viscosity cement paste, which can be applied by injection from a syringe [108]. Currently, injection appears to be the preferred method between these two major options. Thus, a trade-off must be found between a high viscosity leading to too high injection forces and a low viscosity increasing the risk of cement extravasations. Viscosity values in the range of 100 – 2000 Pa·s are generally considered to be adequate [222].

In any case, before using a surgeon needs to have a cement powder and a liquid be mixed properly and thoroughly (to avoid the powder/liquid encapsulation) within the prescribed time and be performed in a sterile environment. Therefore, a mixing procedure is very important

because prior to be injected, a cement paste must be transferred from a mixing chamber into a syringe. Ideally, this should be done without trapping air bubbles by the cement paste [223]. Earlier, most calcium orthophosphate cements were manually mixed with aqueous solutions using a mortar and either a pestle or a spatula. That time, some concerns were raised about an insufficient and inhomogeneous mixing thus compromising the implant strength, as well as on inconsistencies between operators causing unpredictable variations in graft performance [224]. Mechanical mixing (*e.g.*, by either an electrically powered mixing machine of Norian SRS/CRS[®] or Mini-malax[®] mixing system for Cementek[®] cement, produced by Teknimed S.A.) is the modern approach. It allows mixing the cement paste within 60 – 80 s and enables a rapid and reliable filling of the application syringe [30]. Besides, a cement powder and an aqueous solution might be placed into a syringe and mixed inside a shaker to produce a consistent cement paste of the desired viscosity [223]. The mechanical mixing was found to decrease both the mean viscosity of the curing cement paste and variability in the viscosity at a given time [225]. However, it did not improve the mechanical strength of the cement [167, 168].

Of the cements, listed in Table 2, Norian SRS[®] is sold as a reactant pack containing two components: a mixture of dry powders (MCPM + α -TCP + CaCO₃) and a liquid (aqueous solution of Na₂HPO₄). The components are mixed in the operating room. The paste that is formed is malleable and injectable for ~ 5 minutes; it hardens within ~ 10 minutes after injection [21, 175]. However, data are available that out of 4.5 ml Norian SRS[®] cement paste ~ 3 ml is injectable only, whereas up to 1.5 ml of the cement might remain uninjectable from the syringe [25]. This phenomenon is prescribed to the cement rheology and its interaction with the hydraulic forces of the syringe. α -Bone Substitute Material (α -BSM[®]) is also a two-component system; it is prepared from a mixture of ACP and DCPD powders and a saline solution [140]. Biopex[®] consists of four different calcium orthophosphates: 75 wt. % α -TCP, 18 wt. % TTCP, 5 wt. % DCPD and 2 wt. % HA. The aqueous solution contains 12 wt. % sodium succinate and 5 wt. % sodium chondroitin sulfate [226]. Effects of liquid phase on the basic properties of Biopex[®] were investigated. When mixed with neutral sodium hydrogen orthophosphate or succinic acid disodium salt solution, the initial setting times of the cement were 19.4 ± 0.55 and 11.8 ± 0.45 minutes respectively. These setting times were much shorter than that of distilled water, 88.4 ± 0.55 minutes [227]. Biopex[®] is mixed with a spatula inside a syringe that can be opened from the front. After mixing, the front part is closed, a needle is inserted into this front part and the cement paste can be manually injected [167, 168].

Some systematic studies on the influence of composition and concentration of the liquids used in preparing of calcium orthophosphate cements were performed as well [22, 195]. Unfortunately, the results appeared to be rather unclear. For example, for several cements, mixing with sodium citrate or citric acid resulted in some effects on the initial setting time [22, 196], while for other cements the effect was insignificant [195]. Concentration increasing of sodium citrate solution resulted in initial setting time increasing [22, 195], although the injectability variations of the cement pastes were inconsistent [22, 196].

Good injectability, adequate viscosity and satisfactory cohesion are required for the successful biomedical applications of calcium orthophosphate cements [228, 229]. Injectability is the ability to be extruded through a small hole of a long needle (*e.g.*, 2 mm diameter and 10 cm length) [230, 231] (other needles are also applied [232, 233]); and for certain applications, injectability is even a prerequisite. It is measured by the weight percentage of the cement paste that could be injected without demixing from a standard

syringe by either a hand or a force of 100 N maximum (Figure 3). Usually, injectability of a cement paste varies inversely with its viscosity, the P/L ratio, as well as the time after starting the mixing of liquid and powder [48, 231, 234]. When put under pressure, some calcium orthophosphate cements show demixing into a thin paste, which is extruded, and a thick mass, which remains inside the syringe (see the aforementioned example for Norian SRS[®] [25]). The phenomenon, in which the pressure applied to the cement paste provokes a phase separation after a certain injection time, is called filter pressing; the liquid comes out without the particles [230]. Possible mechanisms underlying the limited injectability of hydraulic calcium orthophosphate cements have been discussed in literature [233]. In the case of demixing, the exact composition of the extruded part of the paste becomes unknown. Moreover, due to a deviation from the initial P/L ratio, it becomes unclear whether the setting behavior and the mechanical and histological properties of the extruded part are still clinically acceptable. Therefore, a good cohesion of the paste is necessary in order to avoid these problems [235].

An appropriate cohesion was achieved when no disintegration of the cement paste was observed in the fluid [108, 235]. This can be accomplished by keeping a high viscosity for the cement paste [21] or using cohesion promoters (*e.g.*, 1 % aqueous solution of sodium alginate [147, 236, 237] and other chemicals [147, 238]). Some calcium orthophosphate cements fulfill both criteria, *e.g.*, Norian SRS[®], but others fulfill only one or even none of these requirements. For example, BoneSource[™] [90] and Cementek[®] are not injectable and blood must be kept away from the implanting site until setting [167, 168]. A poor cohesion has been associated to a poor biocompatibility and might lead to inflammatory reactions [239]. Further details on the cohesion properties of various calcium orthophosphate pastes are available in literature [235].

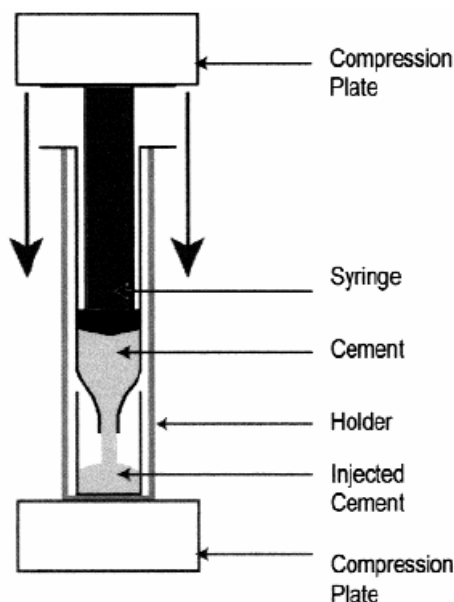


Figure 3. A schematic representation of the experimental setup used to quantify the injectability of the calcium phosphate cements. Reprinted from Ref. [234] with permission.

Several ways can be adopted to improve both the *in vitro* properties and behavior of calcium orthophosphate cements. The first approach consists of injectability improvement. There are two options for this. Firstly, the injection device can be modified. For example, shorter cannulas with a larger diameter, as well as smaller injection rates favor a good injectability. The last option is not so straightforward: for example, Habib *et al.* have shown that large injection rates are not detrimental to injectability because of the shear-thinning behavior of many calcium orthophosphate cements [233]. Secondly, the cement composition can also be adapted. Namely, a decrease of the particle size, the P/L ratio and the plastic limit was found to contribute to a better injectability [230, 234]. For example, injectability was found to be unaffected by P/L ratio within the range of 3.85 – 4.50 g/ml but drops by nearly 100 % between P/L ratio of 4.50 and 5.00 g/ml [22]. However, a decrease in P/L ratio leads to a decrease in the mechanical properties of the cements and the cohesion might be destroyed. Furthermore, both the initial and final setting times decreased markedly with the P/L ratio increasing [195, 240]. Therefore, variations in the P/L ratio appear to be valid to a certain extent only. That is why the manufacturer of Biopex[®] suggests using a P/L ratio of 2.8 or 3.3 g/ml.

Particle size decreasing of calcium orthophosphate crystals is the second approach for the injectability improvement. For example, α -BSM[®] is well injectable because it consists of small particles. Even though small particles require a larger amount of mixing liquid to obtain a paste, injectability and cohesion of the cements are generally very good [167, 168]. An indirect approach is to add calcium orthophosphate particles those act as spacers between other particles. For example, DCPA is added to the formulation of Biocement D[®] to improve the injectability of the paste [167, 168]. Similarly, there is an apatite cement containing spherical particles of TTCP to improve injectability [241].

Using various additives is the second way to improve the physical properties of calcium orthophosphate cements [242]. For example, water demand of calcium orthophosphate cements can be reduced by ionically modifying the liquid component, *e.g.*, by adding nontoxic sodium salts of α -hydroxy di- and tri- acids [243, 244]. A list of additives, that have been already studied, includes fluidificants, air-entraining agents, porogens, workability-improvement agents, setting time controllers and reinforcing additives [135, 173, 245]. Besides, radiopacifiers might be used [246]. The main role of a fluidificant is to reduce a mixing time of the cement. Citric acid is an example of this reagent; it retards the dissolution-precipitation reactions in the cement, decreases the compressive strength during initial setting, but increases its strength in the final stages of the cement hardening [196]. Furthermore, data are available, that citric acid decreases the setting time and improves the mechanical properties of the hardened cements [247]. Air-entraining agents (*e.g.*, surfactants) are commonly used to induce macroporosity inside calcium orthophosphate cements without affecting their normal setting. For example, crystals of mannitol, $\text{CH}_2\text{OH}(\text{CHOH})_4\text{CH}_2\text{OH}$, were tested as an air-entraining agent; however, both loss of workability during the cement mixing and severe depreciation of mechanical properties were discovered simultaneously [248]. Various porogenic agents (*e.g.*, oxygen peroxide [249] in the liquid phase and/or iced [250], sucrose granules, NaHCO_3 and Na_2HPO_4 crystals of 125-250 μm in size [251], poly(DL-lactic-co-glycolic acid) microparticles with the average size of $66 \pm 25 \mu\text{m}$ [252-257], calcium sulfate [40], NaCl crystals varying in size from 420 μm to 1 mm [258, 259], gelatin microspheres [260, 261], cetyltrimethyl ammonium bromide [262], polytrimethylene carbonate [263], some immiscible liquids) have been also tested to create porosity. These

additives could be applied on pre-set cements only. After cement hardening, dissolution of the aforementioned soluble porogens in either water or body fluids produces macropores with the dimensions and shapes of the dissolved crystals. Another method consisting in adding solid NaHCO_3 to the starting cement powder and using two different liquids: first, a basic liquid to form the paste and later an acid liquid to obtain CO_2 bubbles to create the porosity is also available [264]. Besides, pore forming CO_2 bubbles appear at hardening of an apatite cement, consisting of an acidic calcium orthophosphate and either CaCO_3 [29, 45-47] or NaHCO_3 [265-267]. Furthermore, addition of an effervescent porogen formulation comprised from NaHCO_3 (54.52 %) and citric acid monohydrate (45.48 %) has been suggested [268]. Adding of surfactants to calcium orthophosphate cements was found to have two different meanings: they might act as both air-entraining agents by lowering the surface tension [269] and interaction modifiers by shifting the isoelectric point [270].

The major examples of workability-improvement agents, which are added to the cement powders, include water-soluble polymers. Specifically, polysaccharides [84, 95, 271-274], gelatin [240, 275-281] and polyacrylic acid [282-284] are of an interest due to their biocompatibility and good rheological properties. Only small amounts (a few weight %) are needed to dramatically increase the viscosity of the cement pastes. Besides, the cement paste becomes more cohesive and highly resistant to washout immediately after mixing. For example, a 5 wt. % sodium chondroitin sulfate solution is used as mixing liquid in Biopex[®] [167, 168]. In the case of gelatin, more than a 50 % improvement of the compressive strength was detected [277]. The gelatin-cement after setting was found to exhibit reduced crystallinity, much smaller CDHA crystals and a more compact microstructure; all these phenomena might be accounted for the improved mechanical properties [278]. The presence of gelatin improved mechanical properties of the cements; in particular, calcium orthophosphate cements containing 2 wt. % gelatin were found to harden in an acceptable time and were recommended for clinical applications [281]. The use of gelling agents widened a possible application of calcium orthophosphate cements because these cements can be used even when complete homeostasis is difficult. In some cases addition of a gelling agent might cause an increase in hardening time but this was remedied by the use of a sodium orthophosphate solution as the cement liquid [117, 118]. Most polysaccharide solutions are thixotropic, *i.e.*, the viscosity of the solution decreases as the shear rate increases. Certain polysaccharides, such as sodium alginate, pectize in contact with calcium ions. This property can be used to make putty-like cement pastes [21]. However, only few polysaccharides are accepted for parenteral use [167, 168].

Of two families of calcium orthophosphate cements, the brushite cements react generally much faster than apatite ones. As a result, to satisfy the clinical requirements (Figure 2), the setting time of brushite cements has to be prolonged, whereas that of apatite cements has to be shortened [167, 168]. In general, setting reactions of any calcium orthophosphate cements consist of three successive stages: (1) dissolution of reactants to saturate the mixing liquid in calcium and orthophosphate ions; (2) nucleation of crystals; (3) growth of crystals. Therefore, experimental approaches to modify the setting reaction of calcium orthophosphate cements are to be targeted to these three stages. The available approaches have been summarized in Table 3 [176]. Furthermore, seven strategies have been described to control the setting time of calcium orthophosphate cements [177]. They are: (i) mean particle size decreasing of the initial powders; (ii) the P/L ratio increasing; (iii) pH drop of the mixing liquid to increase calcium orthophosphate solubility and hence accelerate the chemical transformations; (iv) a

nucleating phase addition, such as a nanosized HA powder; (v) adding orthophosphate and/or calcium ions into the mixing liquid to accelerate the setting reaction according to the common-ion effect; (vi) solubility reducing of the reaction end-product, for example, by adding fluoride ions into the mixing liquid; (vii) solubility increasing of the starting material by amorphization, *e.g.*, by prolonged milling. For further details on these strategies and approaches, as well as for application examples, the interested readers are referred to the original papers [176, 177].

Various setting time controllers (accelerators and retardants) are used to influence the setting time. They include sodium hydrogen pyrophosphate ($\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$) and magnesium sulfate (according to another study, ions of citrate, sulfate and pyrophosphate are necessary [190]), which are added in amounts < 1 wt. % [285]. Application of biocompatible α -hydroxylated organic acids (glycolic, lactic, malic, tartaric and citric acids) and their calcium and sodium salts for the modification of both rheological and setting properties of calcium orthophosphate cements is well described elsewhere [286, 287]. Besides, aqueous solutions of sodium orthophosphates are also known as setting time accelerators [84, 180, 256, 288-291]. An extensive list of the compounds, which might be suitable as accelerators, retarders, additives or reactants in calcium orthophosphate cement formulations, might be found in literature [86].

The subject of the reinforcing additives is discussed in details below in “Reinforced calcium orthophosphate cement composites and concretes” section.

Table 3. List of strategies and approaches to modify reactivity of calcium orthophosphate cements [176].

Strategy	Approach	Sub-approaches
1. Dissolution rate	1.1. Change contact area between reagent and mixing liquid	1.1.1. Change milling duration
		1.1.2. Use nano- or micro-sized powders
	1.2. Change solubility in the mixing liquid	1.2.1. Use more/less soluble phase
		1.2.2. Change of reaction pH
	1.3. Change saturation of the mixing liquid	
	1.4. Use dissolution inhibitors in the mixing liquid	
	1.5. Modify reagent surface	1.5.1. Chemical change (pre-reaction)
		1.5.2. Physical change (dissolution pits)
2. Nucleation rate	2.1. Use crystallization nuclei	
	2.2. Change the saturation of the reaction product in the mixing liquid	2.2.1. Change of saturation
		2.2.2. Change of end-product solubility
	2.3. Use nucleation inhibitors	
3. Growth rate	3.1. Change the saturation of the reaction product in the mixing liquid	3.1.1. Change of saturation
		3.1.2. Change of end-product solubility
	3.2. Use crystal growth inhibitors	

The factors, that significantly influenced the storage stability (shelf life) of initial dry powders of calcium orthophosphate cements, were found to be temperature, humidity and the mixing regime of the powders. Various storage conditions appeared to be effective in prolonging the stability of dry brushite cements; in the order of effectiveness, they were ranged: adding solid citric acid retardant > dry argon atmosphere $\approx$ gentle mixing (minimal mechanical energy input) >> low temperature [289]. A detailed description of the sterilization techniques for calcium orthophosphate cements might be found elsewhere [292].

5. BIORESORPTION AND REPLACEMENT OF THE CEMENTS BY BONES

Due to the excellent bioresorbability of DCPD and CDHA, a newly forming woven bone might substitute the hardened calcium orthophosphate cements. For example, the implants made of BoneSourceTM were partly resorbed and replaced by natural bone, depending upon the size of the cranial defect [90]. α -BSM[®] was evaluated in a canine femoral slot model. New bone was found to form in 3 weeks via an osteoconductive pathway. After 4 weeks, only 1.7 % of the implanted material was observed. The hybrid bone possessed the strength of normal, unoperated bone after 12 weeks. In 26 weeks, the boundary between old and new bone was virtually indistinguishable, with only 0.36 % of the implant recognizable [140]. Norian SRS[®] was evaluated in canine tibial and femoral metaphyseal defects. The cement appeared to be gradually remodeled over time, with blood vessels penetrating through it. However, some amounts of Norian SRS[®] were detected in the medullary area as long as 78 weeks after being implanted in dog femurs [28]. An interesting study on the *in vitro* resorption of three apatite cements (conventional, fast-setting and anti-washout) by osteoclasts if compared with similar resorption of sintered HA and a cortical bone revealed an intermediate behavior of the cements: they were resorbed slower than bone but faster than HA [293]. Evidences of the direct contact of bone and a calcium orthophosphate cement without soft tissue interposition might be found in literature [294].

Different studies reported on both cement bioresorption and the progress of bone formation around calcium orthophosphate cements which in certain cases demonstrated both osteoconductive and osteoinductive properties [295]. However, some studies did not confirm the osteoinductive properties of calcium orthophosphate cements [296]. Some inflammatory reactions were noticed when the cement did not set [239]. As solubility of a non-stoichiometric CDHA is higher than that of stoichiometric HA, α - and β -TCP (Table 1) and the particle dimensions of precipitated CDHA is smaller than those of sintered calcium orthophosphates, the biodegradability of apatite cements is always better than that of dense bioceramics made of sintered stoichiometric calcium orthophosphates. For example, histologically, at 2 weeks, spicules of living bone with normal bone marrow and osteocytes in lacunae could be seen in the cement. At 8 weeks, the cement was almost totally surrounded by mature bone. At this stage, no resorption of the cement was observed [297]. Only 30 % decrease of the implanted amount of Norian SRS[®] was reported after 24 months in a rabbit femur [298]. Moreover, several differences can be expected depending on the cement type. For example, as the end-product of BoneSourceTM and Cementek[®] is a very crystalline CDHA, BoneSourceTM and Cementek[®] are expected to resorb slower than other apatite cements. Indeed no resorption of BoneSourceTM was observed after several years

implantation; though some resorption of Biobone[®] was detected. However, porosity appears to be the main biodegradability factor at play: a more porous (for cells) hardened cement degrades faster than a less porous one. For example, as Biobone[®] is more porous than BoneSource[™], the discovered diversity could be due to the differences in the cement porosity [167, 168]. The latter conclusion is confirmed by the results of other studies: a positive influence of the cement porosity on the resorption rate was found [237]. The interested readers are referred to the study on the suitability of porous calcium orthophosphate cements as scaffold material for bone regeneration, using a rabbit model [299].

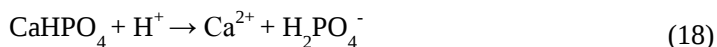
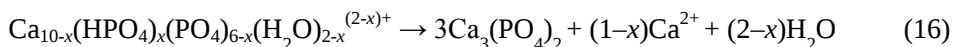
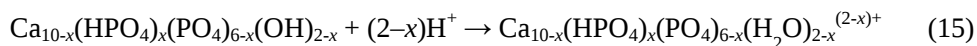
The resorption properties of bioceramics are generally believed to relate to the solubility of their constitutive phases. The implanted calcium orthophosphates might be resorbed by two possible mechanisms, namely: an active resorption, mediated by the cellular activity of macrophages, osteoclasts and other types of living cells (so called phagocytosis or literally “cell-eating”) [300-302] and a passive resorption due to either chemical dissolution [11] or chemical hydrolysis (brushite cements only) [198] in the body fluids. Unfortunately, the factors concerning the biodegradation of calcium orthophosphate biomaterials have not been completely elucidated yet. The chemical composition, physical characteristics and crystal structures certainly play an important role in the biological behavior of calcium orthophosphates. In addition to this, the biodegradation may be influenced by the experimental conditions: experimental models, implantation sites and animal species [301].

The data are available that macrophages and giant cells decompose quickly resorbed calcium orthophosphate cements (*e.g.*, brushite cements) [194], while slowly (from months to years) resorbed apatite cements are decomposed by osteoclast-type cells [26, 178, 303]. Clearly, a fast resorption of brushite cements can only be achieved if the cement resorption occurs before its conversion to CDHA according to equation (14) [41]. Both types of the resorption mechanisms (active + passive) might occur almost simultaneously, if a hardened cement consists of two different calcium orthophosphates, *e.g.*, from DCPD and β -TCP. For example, the biphasic brushite cement ChronOS[™] Inject was found to resorb by dissolution with cement disintegration and particle formation followed by the phagocytosis of the cement particles through macrophages [304]. A similar cement was found to be degraded through a dissolution process associated with a cellular process. The observations suggested that cell activities could be influenced by a small particle size, without close correlation between the particle size and the cell activities but with a correlation between particle concentration and the cell activities [301]. The interested readers are referred to a very interesting review on the cellular mechanisms of calcium orthophosphate ceramic degradation [305].

The summary of brushite cement implantation studies in various animal models and defect locations is available in literature [198]. Generally, in the same animal model, a degradation rate decreases with a sample size increases, as does DCPD to HA conversion time. The compositional changes of a brushite cement after implantation in sheep is well described elsewhere [285, 306].

The kinetics of passive resorption depends on porosity of the samples, ionic substitutions, crystallinity and pH of the cement-tissue interface. The active resorption is due to cellular activity; however, it is also related to the passive one. Solution pH near macrophages and osteoclasts can drop to ~ 5 by the excretion of lactic acid, whereas near osteoblasts (bone forming cells) solution pH can become as high as 8.5 by the excretion of ammonia [25]. Dissolution chemistry of CDHA (therefore, of the hardened apatite cements) in acidic media

(calcium orthophosphates are almost insoluble in alkaline solutions [10, 11, 189]) might be described as a sequence of four successive chemical equations [307, 308]:



Obviously, the dissolution chemistry of DCPD (therefore, of the hardened brushite cements) in acidic media is described by equation (18). One should stress, that in equation (18) water is omitted for simplicity. Therefore, dissolution of DCPA is written instead.

The mechanism of bone healing caused by calcium orthophosphate cements is very multifactorial because the surface of the cements is rapidly colonized by cells. Several types of these cells degrade calcium orthophosphates by either phagocytotic mechanisms (fibroblasts, osteoblasts, monocytes/macrophages) or an acidic mechanism with a proton pump to reduce the pH of the microenvironment and resorb the hardened bioceramics (osteoclasts) [305, 309]. Various mesenchymal cells located at the implantation sites can induce solubilization of calcium orthophosphates. Upon the cells arrival, various active enzymes, such as acid phosphatase, are secreted that causes dissolution of the hardened cements [310-312]. Much more biology, than chemistry and material science altogether, is involved into this very complex process and many specific details still remain unknown. Due to a lack of the necessary experimental data for calcium orthophosphates, the major bone healing steps caused by the cements might be schematically described by a modified scheme for the bioactivity mechanism of bioactive glasses – the concept introduced by Prof. Larry L. Hench [313, 314]. The mechanism of bonding of bioactive glasses to living tissue involves a sequence of 11 successive reaction steps. The initial 5 steps occurred on the surface of bioactive glasses are “chemistry” only, while the remaining 6 steps belong to “biology” because the latter include colonization by osteoblasts, followed by proliferation and differentiation of the cells to form a new bone that had a mechanically strong bond to the implant surface (Figure 4).

It is well known that various polypeptides and growth factors present in bone matrix might be adsorbed onto HA and modulate the local milieu of cells. This is supported by many purification protocols of growth factors and bone morphogenetic proteins/osteogenins involving HA chromatography [316, 317]. However, osteoblasts are not found in direct contact with calcium orthophosphates. A complex proteinaceous layer, usually osteoid, directly contacts the osteoblasts. After implantation of calcium orthophosphate cements, mitogenic events could occur either during the initial mesenchymal cell contact or after osteoid degradation by osteoblast collagenase. In a dense, mineralized material such as calcium orthophosphate cements, which provides a barrier to the free diffusion of circulating hormones, growth factors, and cytokines, it is questionable whether the local responses at the periphery of the material regulate osteoconduction [21]. The tissue response to injectable calcium orthophosphate cements is well described in literature [265, 293, 303, 318, 319].

Recent histological and mechanical evaluation of self-setting calcium orthophosphate cements in a sheep vertebral bone void model is available elsewhere [320]. The interested readers are also advised to get through a recent paper on the *in vitro* biodegradation of brushite cements by a macrophage cell-line [105].

To conclude this part, one should note that calcium orthophosphate cements are able to provide short-term biologically desirable properties and then be replaced by a new bone, which is very important [321]. The growth rate of a newly forming bone depends on age, sex and general metabolic health of the recipient as well as on the anatomic site, porosity, bulk site, crystallinity, chemical composition (brushite or apatite), particle sizes and P/L ratio of the cements. Considering all these factors, it might take from 3 to 36 months for different calcium orthophosphate cements to be completely resorbed and replaced by bone [172]. However, additional sound scientific data to determine the exact degree of biodegradability for calcium orthophosphate cements are still needed, viz. animal studies performed in a critical-size defect model. One must stress that the rate of cement resorption should be balanced with the rate of new bone formation to avoid collapse at the fracture site, which might occur if the resorption is too fast.

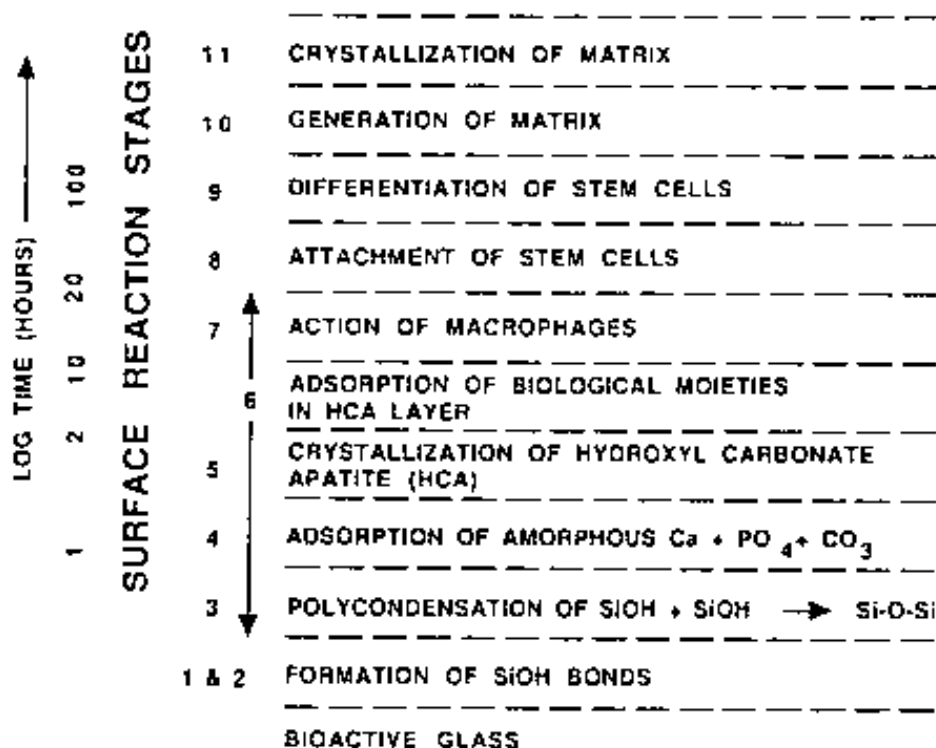


Figure 4. The sequence of interfacial reactions involved in forming a bond between tissue and bioactive glasses. The border between “dead” and “alive” occurs approximately at stage 6. For want of anything better, the bioactivity mechanism of calcium orthophosphate cements should also be described by this scheme with omitting of several initial stages, as it was made for HA in Ref. [315], where 3 initial chemical stages of the Hench’s mechanism were replaced by partial dissolution of HA. Reprinted from Ref. [314] with permission.

6. THE MECHANICAL PROPERTIES

As in most clinical applications calcium orthophosphate cements are applied in direct contact with human trabecular bones, it may be stated as a mechanical requirement that the strength of the cements must be at least as high as that of trabecular bones, which is close to 10 MPa [322]. Three-dimensional (3D) complex load is applied during the orthopedic and dental applications because of a combination of different forces that may include bending, torsion, tension and compression. Unfortunately, calcium orthophosphate cements are strong enough at compression only [165]. In theory, after setting, they can reach the mechanical properties comparable to those of calcium orthophosphate blocks with the same porosity. However, in practice, the strength of the cements is lower than that of bones, teeth or sintered calcium orthophosphate bioceramics [118].

Having the ceramic origin, the set products of all calcium orthophosphate cements are brittle, have both a low impact resistance and a low tensile strength (within 1 to 10 MPa), whereas the compression strength varies within 10 to 100 MPa [115, 165, 166]. The latter value exceeds the maximum compression strength of human trabecular bones. On the other hand, at 12 weeks after implantation the compressive strength of these cements was found to be still significantly higher (60 to 70 MPa) than that of normal bone [31]. Moreover, the mechanical properties of calcium orthophosphate cements are not narrowly distributed around a mean value (as for metals), but widespread over a very large range of values, which strongly reduces their clinical application [323]. Brushite cements are slightly weaker than apatite cements. A tensile strength of 10 MPa and a compressive strength of 60 MPa were obtained for brushite cements [324]. In comparison, apatite cements can reach a tensile strength of 16 MPa [325] and a compressive strength of 83 MPa [326]. *In vivo*, the difference between apatite and brushite cements boosts: namely, the mechanical properties of apatite cements were found to increase [291], whereas those of brushite cements decreased [31]. This is attributed to a higher solubility of DCPD when compared with that of CDHA (Table 1). After a few weeks of implantation, the mechanical properties of brushite cements began to increase due to bone ingrowth [31]. The interested readers are suggested to get through the mechanical characterization of a bone defect model filled with ceramic cements [170].

To improve the mechanical properties of calcium orthophosphate cements, addition of water-soluble polymers might be considered. For example, in early 1990-s, Miyazaki *et al.* [327, 328] used a number of polymers, including polyacrylic acid and polyvinyl alcohol to improve the properties of a TTCP + DCPD cement. They noted marked increases (up to threefold) in mechanical properties but with an unacceptable reduction of workability and setting time. Later, another research group reported similar results using sodium alginate and sodium polyacrylate [329]. Afterwards, other researchers added several polyelectrolytes, polyethylene oxide and a protein bovine serum albumin into α -BSMTM cement paste to create calcium orthophosphate – polymer composites [330]. Composites of α -BSMTM with polycations (polyethylenimine and polyallylamine hydrochloride) exhibited compressive strengths up to six times greater than that of pure α -BSMTM material. Composites of α -BSMTM with bovine serum albumin developed compressive strengths twice that of the original α -BSMTM cement [330]. Similar strengthening effect was achieved by addition of some commercial superplasticizers [331]. The results showed that small additions, *i.e.* 0.5 vol. %, in the aqueous liquid phase improved the maximum compressive strength (35 MPa) of

Biocement-H[®] by 71 %, *i.e.* till 60 MPa. Moreover, the addition of high amounts of superplasticizers, *i.e.* 50 vol. %, allowed for a significant increasing of the P/L ratio from 3.13 to 3.91 g/ml, without affecting the maximum strength and/or the workability of the cement [331]. This effect was explained by an inhibiting effect of the aforementioned additives on the crystal growth kinetics of newly forming crystals of calcium orthophosphates, which resulted in smaller crystallites and, hence, a denser and more interdigitated microstructure. However, the increased strength was attributed mainly to the polymer's capacity to bridge between multiple crystallites (thus forming a more cohesive composite) and to absorb energy through a plastic flow [330].

As presence of pores simplifies for cracks to run throughout the hardened mass, the mechanical properties of the hardened cements were found to decrease exponentially with the porosity increase [332]. In theory, calcium orthophosphate cements can be made with almost any porosity. However, for most commercial cements, the pores are of 8 – 12 µm in diameter and, after the cement is set, about 40 – 50 % of its volume is the porosity [333]. Pressure can be applied to reduce the porosity of calcium orthophosphate cements [118, 334, 335]. The pore dimensions in hardened cements are too small to allow fast bone ingrowth. There is a lack of macroporosity. Besides, unless the special efforts have been performed, the available macroscopic pores are not interconnected. Due to these reasons, after injection, osteoclastic cells are able to degrade the hardened cements layer-by-layer only, starting at the bone-cement interface throughout its inner part (in other words, from the outside to the inside). This is the main drawback of the classical cement formulations when compared to calcium orthophosphate ceramic scaffolds with an open macroporosity [167, 168].

Since the compression strength is reciprocally proportional to porosity, the former might be adjusted by varying the P/L ratio in the hardening mixture. Elevated compression strength would be applicable in cranioplasty for regions requiring significant soft-tissue support. For smaller bone defects, such as root canal fillings, low-compression cements might be used [111]. Concerning the tensile strength of calcium orthophosphate cements, as a rule of thumb, it appears to increase two-fold with each 10 vol. % decrease of the porosity, *i.e.* 5, 10, 20, 40 and 80 MPa for 80, 70, 60, 50 and 40 % porosity, respectively [167, 168]. The effect of porosity on the compressive modulus of calcium orthophosphate cements is available as Figure 4 in Ref. [335]. Ishikawa and Asaoka showed a linear relation ($R^2 = 0.94$) between $\ln$ diametral tensile strength and porosity of a calcium orthophosphate cement where porosity was controlled by compaction pressure (up to 173 MPa) [115]. Besides, an empirical relationship between strength, S , and porosity, P is also available [336]:

$$S = S_0 e^{-bP}$$

where: S_0 is the theoretical strength at $P = 0$ (fully dense) and b is an empirical constant.

As the porosity is mainly due to an excess of water used in the cement compositions, attempts were made to reduce the amount of water. Besides, the amount of water determines the rheological properties of the cement paste: a decrease in water content leads to a large increase in viscosity, eventually leading to non-flowable pastes. As calcium orthophosphate cements set at an almost constant volume, the final porosity can be predicted from the initial composition [167, 168]. A shrinkage degree of ~ 1 % causes no restrictions on clinical use [163]. Recent studies on the *in vivo* evaluation of an injectable macroporous calcium orthophosphate cements revealed a higher bioresorption rate due to both a higher surface

contact with body fluids (which increases dissolution) and enhancing cellular activity due to particle degradation [237, 265].

According to Bohner [167], it is difficult to compare the mechanical properties of different cement formulations. For example, the following numeric values of the compression strength and setting time were obtained for Norian SRS[®]: 33 ± 5 MPa and 8.5 ± 0.5 min (≈ 50 % porosity), Cementek[®]: 8 ± 2 MPa and 17 ± 1 min, Biocement D[®]: 83 ± 4 MPa and 6.5 ± 0.5 min (≈ 40 % porosity) and α -BSM[®]: 4 ± 1 MPa and 19 ± 1 min (≈ 80 % porosity), respectively [326]. Among them, Biocement D[®] has the highest compressive strength but the lowest porosity. A high compressive strength does not necessarily mean that Biocement D[®] is the least breakable implant. *In vivo*, shear and tensile forces indeed play a very important role. Therefore, the tensile strength of the cements should also be considered, for example, using the Mohr circle approach [337]. Finally, it should be kept in mind that the initial mechanical properties of calcium orthophosphate cements may vary with implantation time. Animal studies indicated that the mechanical properties of apatite cements tended to increase continually [291], in contrast to those of brushite cements, which initially decreased and again increased when bone was growing [31]. Further details on the major properties of Norian SRS[®] are available elsewhere [173, 338].

The porosity level of calcium orthophosphate cements might be controlled to a certain extent by adjusting particle sizes and the P/L ratio. When the P/L ratio is high, the porosity of the apatite cement is low [167, 168]. Besides, successful attempts have been made to introduce macroporosity into calcium orthophosphate cements by using soluble particles (porogens) [248, 251, 274, 336, 339], resorbable polymers [340, 341], fast resorptive phases [188, 249] or foaming agents (*e.g.*, dehydrated albumen) [237, 249]. According to calculations, the tensile strength of the cements with zero porosity could be as high as 103 MPa [115]. However, a high density and a lack of pores decreases cement bioresorbability because a newly forming bone appears to be unable to grow into the pores; it might grow only simultaneously with dissolution of the cement. Thus, the porosity of calcium orthophosphate cements is a very important factor for the cement degradability [167, 168]. Other factors affecting strength are the materials used in the solid phase, particle sizes, incorporation of fillers into the solid phase, the P/L ratio and various liquid phases [93].

The strength of the cement-prosthesis interface might be studied by a pullout test. The details are available elsewhere [57].

7. REINFORCED CALCIUM ORTHOPHOSPHATE CEMENT COMPOSITES AND CONCRETES

Being aware on the excellent bioresorbability of DCPD and CDHA, researchers are focused on attempts to overcome the mechanical weakness of calcium orthophosphate cements by using different fillers, fibers and reinforcing additives that give rise to formation of various multiphasic composites [91, 92, 96, 169, 226, 238, 333, 336, 342-347]. Even carbon nanotubes have been successfully tested to reinforce calcium orthophosphate cements [348]. Although the biomaterials community does not use this term, a substantial amount of such formulations might be defined as calcium orthophosphate concretes [349]. The idea behind the concretes is simple: if a strong filler is present in the matrix, it might stop crack

propagation. However, adding fillers always reduced the porosity that negatively influenced the ability of the concretes to allow bone ingrowth into pores. Hence, a denser cement has a slower resorption rate and thus a slower bone substitution [115]. Moreover, due to the presence of fillers, the rheological properties and injectability of calcium orthophosphate concretes frequently appear to be worse than those properties of calcium orthophosphate cements. Thus, it is difficult to increase strength of the cements without having a negative influence on the other properties.

Calcium orthophosphate concretes can be prepared from both apatite and brushite cement formulations. For example, in an attempt to improve the mechanical properties of calcium orthophosphate cements, a group of investigators prepared concretes by adding human cadaveric femur bone chips in amounts of 25, 50 and 75 % (w/w) to α -BSM[®] cement [343]. The mechanical tests revealed that the specimens of pure cement exhibited a relatively high stiffness but a low ductility. However, for the cement-bone concretes an increasing of bone content was found to result in the elastic modulus decreasing and the ductility increasing; however, the ultimate strength showed only small changes with no apparent trend [343]. A concrete of Biopex[®] cement with allografts taken from femurs and tibiae of rabbits is also available. Unfortunately, nothing is written on the mechanical properties improvement but, surprisingly, by the addition of allografts, the hydrolysis process of Biopex[®] was significantly changed [226]. By adding polymers and composites, other researchers succeeded in improving the mechanical strength of the cements up to 30 MPa; however, the kinetics of CDHA formation and thus the bioactivity of the material were decreased [97, 350]. Xu *et al.* reported that incorporation of long carbon fibers at a volume fraction of 5.7 % increased the flexural strength about 4 times and work of fracture 100 times, if compared to un-reinforced calcium orthophosphate cements [351]. The reinforcement mechanisms were found to be crack bridging and fiber pullout, while fiber length and volume fraction were key microstructural parameters that determined the concrete properties [351]. Although addition of polypropylene, nylon and carbon fibers was found to reduce the compression strength of a double-setting calcium orthophosphate cement because of increased porosity, it strongly increased the cement's fracture toughness and tensile strength, relative to the values for the un-reinforced variant of this cement [344]. A knitted two-dimensionally oriented polyglactin fiber-mesh was found to be effective in improving load-bearing behavior of a calcium orthophosphate cement for potential structural repair of bone defects [169]. To make the material stronger, fast setting and anti-washout, chitosan was added to the cements [280, 327, 352-361]. Calcium orthophosphate cements doped by SiO₂ and TiO₂ particles showed a significant (~ 80 – 100 MPa) increase in the compressive strength, whilst no change in the mechanical behavior of the cements was observed when ZrO₂ particles were added [345]. Besides, calcium orthophosphate cements might be successfully reinforced by addition of calcium silicates [59], polypeptide copolymers [362] and collagen [363-365].

Yet another team examined the effects of varying fiber type, fiber length and volume fraction of fiber-reinforced calcium orthophosphate concretes [352, 366]. Four fiber types were studied: aramid, carbon, E-glass and polyglactin. Fiber length ranged from 3 – 200 mm and fiber volume fraction ranged from 1.9 – 9.5 %. The results indicated that a self-setting calcium orthophosphate cement was substantially strengthened via fiber reinforcement. Aramid contributed to the largest increase in composite strength, followed by carbon, E-glass and polyglactin. Fiber length, fiber volume fraction and fiber strength were found to be key microstructural parameters that controlled the mechanical properties of calcium

orthophosphate concretes [352, 366]. Fiber reinforcement of porous cements (mannitol was used as a porogen) was discovered as well [367]. Namely, reinforcement by aramid fibers (volume fraction of 6 %) was found to improve the properties of a calcium orthophosphate cement with the strength increasing threefold at 0 % mannitol, sevenfold at 30 % mannitol and nearly fourfold at 40 % mannitol. Simultaneously, the work of fracture increased by nearly 200 times, however the modulus was not changed as a result of fiber reinforcement [367]. Addition of 20 wt. % of acrylamide and 1 wt. % ammonium polyacrylate to the liquid increased the compressive and tensile strength of α -TCP bone cement by 149 and 69 % (55 and 21 MPa), respectively [368]. A positive influence of polyamide fibers [369] and bioactive glass [370] is also known.

In the cases, when bioresorbable reinforcement fibers are used, strength augmentation is attained at the initial stages [340, 371-374]. For example, the initial strength of a concrete was threefold higher than that of the unreinforced cement control [371]. The work of fracture (toughness) was found to increase by two orders of magnitude for other composites of calcium orthophosphate/resorbable fiber (namely, Vicryl polyglactin 910, Ethicon, Somerville, NJ [372] and a mesh of copolymer of polyglycolic and polylactic acids [340]). When implanted *in vivo*, bioresorbable fibers would provide initial strength and then dissolve to form interconnecting macroscopic channels, which could facilitate bone ingrowth into the implant [117, 118, 340, 371]. For example, interconnected macropores were formed in a calcium orthophosphate cement at 84 days' immersion in a physiological solution [340]. One should note that, apart from the mechanical properties of the reinforcing material, the structure of the incorporated fibers, regular or random, appears to be crucial for the resulting flexural strength and modulus of elasticity [374]. A higher strength might help extending the use of calcium orthophosphate cements to larger stress-bearing repairs, while the macropores might facilitate tissue ingrowth and integration of the cement with an adjacent bone. To extend this idea further, several types of fibers with different rates of bioresorbability might be simultaneously incorporated into a cement formulation.

Besides the aforementioned, it is important to mention on concretes, after hardening consisting of calcium orthophosphates only [205, 304, 375-378]. The first biphasic composition consisting of a hardened DCPD matrix filled with β -TCP granules was introduced in 1992 [376]. Further development of this formulation might be found in other papers [205, 304]; unfortunately, neither the mechanical nor the rheological properties of this concrete have been disclosed. At physiologic pH, the *in vitro* solubility of DCPD is approximately 100 times higher than β -TCP; roughly, the same order of magnitude applies for the *in vivo* resorption kinetics of these calcium orthophosphates. A new bone forms in the space left after resorption of the DCPD matrix, while β -TCP granules act as guiding structures. This feature of the cement can be considered an inverse scaffolding effect [379]. Another group of investigators invented a formulation that incorporated as major powder components α -TCP, ACP and biphasic calcium phosphate (BCP; consisting of an intimate mixture of HA and β -TCP in various HA/ β -TCP ratios) [342]. It was believed that after setting such a formulation could provide a porous ceramics *in vivo* due to preferential dissolution of a better soluble ACP component compared to the other calcium orthophosphates in the matrix. Further, this combination was extended to a multiphase concrete composition consisting of 70 % w/w settable matrix (mixture of 45 % α -TCP, 5 % MCPM and 25 % ACP [380]) with the average particle dimensions of 15 μ m and 30 % BCP granules (ranging between 80 and 200 μ m) as a filler [375]. The role of BCP granules is quite

interesting: after implantation of a cement without BCP granules, the quality of newly formed bone was not identical to the host bone, while implantation of a concrete with BCP granules resulted in formation of a new bone identical to the host bone. The reason of this phenomenon is not clear yet; but, perhaps, it correlates with similar results for β -TCP granules, which act as bone anchors and encourage formation of a mature bone [205, 206].

Effects of added α -TCP and β -TCP were investigated to shed light on the setting reaction of apatite cement consisting of TTCP and DCPA [378]. Added β -TCP showed no reactivity, and thus resulted in extended setting time and decreased mechanical strength. In contrast, α -TCP dissolved to supply calcium and orthophosphate ions after initial apatite crystal formation by the chemical reaction (1). Although setting time was delayed because α -TCP was involved only in the latter reaction of apatite cement, larger apatite crystals were formed due to its addition. Because of larger apatite crystal formation, the mechanical strength of α -TCP-added apatite cement increased by approximately 30 %, as compared to α -TCP-free apatite cement [378].

A strength improvement was found when DCPA and TiO_2 crystals were used as fillers for mechanically activated α -TCP cements [381]. Calcium orthophosphate concretes reinforced by whiskers made of calcium carbonate [47] and HA [377] have been also developed.

To conclude this part, one should briefly mention on the reverse situation: there are bone concretes made of acrylic cements, reinforced by calcium orthophosphate powders or granules [382-387]. The calcium orthophosphates presented in these formulations act as fillers, which are necessary to improve the mechanical properties and to impart bioactivity; they do not participate in the hardening mechanisms. Polymerization of monomers is primarily responsible for setting of such composites and concretes. However, that is another story.

8. CLINICAL AND MEDICAL APPLICATIONS

Injectable osteoconductive calcium orthophosphate cements have been introduced as an adjunct to internal fixation for treating selected fractures. Different studies have already shown that they are highly biocompatible and osteoconductive materials, which can stimulate tissue regeneration [21, 389]. The main purpose of calcium orthophosphate cements is to fill voids in metaphyseal bone, thereby reducing the need for bone graft, although the cements also might improve the holding strength around metal devices in osteoporotic bone. Bone augmentation (*i.e.*, a reinforcement of osteoporotic bone through injection) appears to be a very promising application field of calcium orthophosphate cements. Such procedures ease the fixation of screws in mechanically poor bone (for example for osteosynthesis) and decrease pains associated with unstable vertebrae. The combination of a self-setting nature, moldability, biocompatibility, lack of any by-products and a great potential for being replaced by bone make calcium orthophosphate cements very promising materials for clinical applications: they can easily be used by bone remodeling cells for reconstruction of damaged parts of bones [89, 90, 194, 319, 390, 391]. The ability to be molded in place also is a very important property, because a cement can easily be delivered into the desired place and can be fitted perfectly with bone defects [90]. Besides, some formulations were found to possess an

antimicrobial activity [48, 51, 53, 60, 392], as well as promote osteoblast cell adhesion and gene expression *in vitro* [393].

Recent studies reported optimistic results in relation to the clinical application of calcium orthophosphate cements. For example, the data on cytocompatibility and early osteogenic characteristics are available in literature [394]. The ratio of the cases determined to be “effective” or “better” among the 74 cases we found to be 97.3 % [395]. Besides, the results of intra-articular degradation and resorption kinetics of these cements revealed no signs of pronounced acute or chronic inflammation [396]. Injected Norian SRS[®] cement was mainly found as a single particle, anterior to the cruciate ligaments. The cement became surrounded by synovial tissues within 4 weeks and showed signs of superficial resorption [396]. Unfortunately, disintegration or washout of calcium orthophosphate cements has been reported as a potential clinical problem [115, 179]. Perhaps, this problem could be solved by putting pressure on the paste during the setting period. In addition, sodium alginate might be added; however, the mechanical properties (strength) of this formulation are still poor [95].

According to the available information, the first animal study of calcium orthophosphate cements was performed in 1987 [126]. Afterwards, in 1991, a cement consisting of TTCP and DCPA was investigated histologically by implanting disks made of this cement within the heads of nine cats [397]. Simultaneously, another research group evaluated the tissue reaction to this cement in the teeth of monkeys [398]. The important examples of the most significant directions of current medical applications of calcium orthophosphate cements and concretes are given below.

8.1. Dental Applications

A group of investigators extracted all mandibular premolar teeth from beagles [399]. After one month of healing, alveolar bone was reduced to make space for a previously fabricated calcium orthophosphate cement block. One more month later, 8-mm HA implants were placed in such a manner that the apical half was embedded into alveolar bone and the coronal half in the calcium orthophosphate cement block. The investigators observed that the cement block was gradually replaced by bone and histopathologic features of the cement area were similar to that of natural bone. Moreover, the coronal half of the implants, previously surrounded by the calcium orthophosphate cement, was firmly attached by natural bone [399]. In another study, the same researchers used fluorescent labeling analysis and electron microanalysis to measure the extent of new bone formation and elemental (Ca, P, Mg) distribution [400]. The results indicated the presence of newly formed bone at 1 month after surgery and similar elemental distributions in the calcium orthophosphate cement and natural bone areas at 6 months after surgery [172]. Besides, calcium orthophosphate cements were tried as root canal fillers [51, 401, 402] and for pulp capping [403].

A hydraulic calcium orthophosphate cement was injected as a bone filler for gaps around oral implants placed on the medial femoral condyles of six goats and found excellent bone formation around the graft material. Unfortunately, the degradation rate of the cement appeared to be very slow and no resorption was observed [404]. In another study, a cement was placed on artificially created periodontal defects but no significant difference was found between the cement and control. However, the cement acted as a scaffold for bone formation and provided histocompatible healing of periodontal tissues [405]. Still other investigators

used a cement for direct pulp capping and compared it to calcium hydroxide. Both materials were found to be equally capable of producing a secondary dentin at 24 weeks [406].

8.2. Craniofacial and Maxillofacial Applications

The use of calcium orthophosphate cements for craniofacial applications seems logical, as there is little or no stress generated under these conditions. Moreover, the ability to mold the material at placement is an enormous advantage from a cosmetics standpoint [172]. For example, BoneSourceTM is indicated for the repair of neurosurgical burr holes, contiguous craniotomy cuts and other cranial defects with a surface area no larger than 25 cm² per a defect. In addition, it may be used in the sinus region for facial augmentation [90, 407] and the cement can be supported by metal hardware [90]. In dogs, BoneSourceTM was employed to supplement the supraorbital ridge and to augment skull base defects [408]. Another group performed trials to ascertain the inflammation around the site and the degree of loss of the implanted BoneSourceTM. The material was found to be osteoconductive with both periosteal and endosteal bone formation [409]. One more group presented excellent results using the material combined with an underlying resorbable mesh in calvarian defects of Yorkshire pigs. They found progressive bone ingrowths in all defects at 180 days, with nearly complete replacement by host bone [341]. Besides, excellent results for over 100 human patients were reported when a calcium orthophosphate cement was used in cranial defects. The success rate of the cement after 6 years was 97 % [81]. The results of still other medical trials are available elsewhere [189, 410-417].

8.3. Orthopedic Applications

Calcium orthophosphate cements have successfully been used for treatment of the distal radius fracture [175, 418, 419]. Besides, other successful attempts have been made to use the cements for calcaneal fractures [420], hip fractures [421, 422], augmentation of osteoporotic vertebral bodies [423], tibial plateau fractures [29, 424-427], restoration of pedicle screw fixation [428], reinforcement of both thoracolumbar burst fractures [429], cancellous bone screws [430], in wrist arthrodesis [431] and for fixation of titanium implants [432]. A recent study on a cement augmentation of the femoral neck defect might be found elsewhere [433]. Considering their properties, calcium orthophosphate cements might potentially be applied to reinforce osteoporotic vertebral bodies [423, 434]. Further details are available elsewhere [435, 436]. Besides, calcium orthophosphate cements appear to be a reliable subchondral replacement material when the bone defect is adjacent to the articular cartilage [437].

8.4. Vertebroplasty and Kyphoplasty Applications

Vertebroplasty and kyphoplasty are two surgical procedures that recently have been introduced to medically manage of osteoporosis-induced vertebral compression fractures. Particularly, both procedures aim to augment the weakened vertebral body, stabilize it and/or restore it to as much of its normal height and functional state as possible. Both procedures

involve injection of a self-setting paste of a calcium orthophosphate cement into the fractured vertebral body, which resulted in a faster healing [81, 173, 437-443]. Furthermore, prophylactic injections of calcium orthophosphate cements also have been performed.

8.5. Drug Delivery Applications

In general, a potential substrate to be used as a drug carrier must have the ability to incorporate a drug, retain it in a specific target site and deliver it progressively with time in the surrounding tissues. Additional advantages are provided if the material is injectable, biodegradable, sets at ambient temperature, has near neutral pHs and a large surface area [32, 33]. These properties make calcium orthophosphate cements to be very attractive candidates as drug carriers for therapeutic peptides [445], antibiotics [446-455], anticancer drugs [456], anti-inflammatory drugs [457, 458], cytokines [459], hormones [460] and bone morphogenetic proteins [359, 461-465]. For example, a “growth factor cement (GFC)” has been reported [466]. In that study, a combination of bone morphogenetic protein-2 (BMP-2), transforming growth factor-beta (TGF- β 1), platelet-derived growth factor and basic fibroblast growth factor (bFGF) was used in a calcium orthophosphate cement for treatment of peri-implant defects in a dog model. The findings indicated a significant effect of GFC on increased bone-to-implant contact and amount of bone per surface area if compared with both the cement-only and no-cement treatment groups [466]. Similar data were found for a combination of a calcium orthophosphate cement with an exogenous nerve growth factor [467]. Even more complicated combination of deproteinized osteoarticular allografts integrated with a calcium orthophosphate cement and recombinant human vascular endothelial cell growth factor plus recombinant human BMP-2 (rhBMP-2) has been studied as well [468].

In principle, drugs might be incorporated into both a liquid and a powder phase of the cements. After setting, the drugs are slowly released through the cement pores [179, 451-454, 469, 470]. For example, a group of investigators added flomoxef sodium to a cement formulation and found that the release of antibiotic could be easily controlled *in vivo* by adjusting the content of sodium alginate in the formula [179]. *In vitro* elution of vancomycin from calcium orthophosphate cement has been studied as well [470]. The possibility of using calcium orthophosphate cements as a drug-delivery system offers an attractive and efficient solution for the treatment of various bone diseases, *e.g.*, tumours, osteoporosis and osteomyelitis, which normally require long and painful therapies.

The laboratory studies on drugs incorporation into the cements cover different aspects. Firstly, it is necessary to verify that addition of a drug does not influence the setting reaction not only in terms of the setting and hardening mechanisms but also with respect to the rheological behavior and injectability. Secondly, it is necessary to determine the *in vitro* kinetics of drug release. Thirdly, the drug delivery properties of the cement must be studied *in vivo*. Finally, but still importantly, the clinical performance of the drug delivery system must be evaluated as well [32, 33]. For example, recombinant human transforming growth factor β 1 (rhTGF- β 1) was added to a calcium orthophosphate cement [471-474]. This resulted in formation of a bioactivated cement that could be used as a bone filler and for the replacement of bone [471]. It appeared that after 8 weeks the addition of growth factors stimulated and increased bone formation (50 % volume) and bone contact (65 %) in comparison to control

calvarian defects in an animal study. Besides, the growth factor group reduced the remaining volume of the cement by 20 % [472]. Examples of rhBMP-2 release from a loaded porous calcium orthophosphate cement might be found elsewhere [474, 475], while an experimental study on calcium orthophosphate cement impregnated with dideoxy-kanamycin B is also available [476].

Although most materials currently used as drug carriers are polymers, in the specific field of the pharmacological treatment of skeletal disorders, calcium orthophosphate cements have an added value due to their bioactive character and injectability. Further details and additional examples of the drug delivery application of calcium orthophosphate cements are well described elsewhere [25, 32, 33].

8.6. Brief Conclusions on the Medical Applications

To conclude this part, one should stress that despite several encouraging results, not every surgeon's expectation has been met yet [476]. First of all, calcium orthophosphate cements and concretes are not superior to autografts, despite offering primary stability against compressive loading [477, 478]. One of the main concerns of clinicians is to reach higher rates of bioresorption, an improvement of bone reconstruction and to a lesser extent, higher mechanical resistance [29]. Besides, clinical application of the cements in comminuted fractures revealed penetration of the viscous paste into the joint space [479-481]. The interested readers are referred to a paper on cement leakage during vertebroplasty [482]. To date, cadaveric studies have already shown that using calcium orthophosphate cements with conventional metal fixation in certain fractures of the distal radius, tibial plateau, proximal femur and calcaneus can produce better stability, stiffness and strength than metal fixation alone. Early clinical results have revealed a reduced time to full load bearing when the cements were used for augmentation of tibial plateau and calcaneal fractures, more rapid gain of strength and range of motion when used in distal radius fractures and improved stability in certain hip fractures [391, 418]. However, surgeons reported on difficulties in filling the vertebral bodies (a bad injectability of present formulations) and other problems, such as filter-pressing and cement decohesion, observed during vertebral body injection that resulted in bone instability due to low mechanical strength as well as long setting times of the cements [483]. This happens due to not only low mechanical properties of calcium orthophosphate cements but also some difficulties of filling vertebral bodies. In order to maintain a good cohesion and reduce filter-pressing, calcium orthophosphate cements need to be more viscous (hence, less injectable) [167, 168]. For example, calcium orthophosphate cements might be modified by addition of polysaccharides [84, 95, 271-274] and/or gelatin [240, 275-280].

Another type of concerns has been raised that the use of calcium orthophosphate cements for the augmentation of fractured and osteoporotic bones might aggravate cardiovascular deterioration in the event of pulmonary cement embolism by stimulating coagulation [484]. To investigate these potential problems, 2.0 ml of either calcium orthophosphate or polymethylmethacrylate (PMMA) cement were injected intravenously in 14 sheep. Intravenous injection of calcium orthophosphate cement resulted in a more severe increase in pulmonary arterial pressure and decrease in arterial blood pressure compared to the PMMA cement. Disintegration of the calcium orthophosphate cement seemed to be the reason for more severe reaction that represents a risk of cardiovascular complications. The authors

concluded that further research efforts should aim at improving cohesion of calcium orthophosphate cements in an aqueous environment for future clinical applications such as vertebral body augmentation [484].

To conclude the medical part of this review, one should mention that, although the long-term outcomes are still poorly documented, currently there are no doubts concerning a very great potential of the clinical applications of calcium orthophosphate cements and concretes for healing of bone and dental defects. For example, a bioresorbable calcium orthophosphate cement was once found to be a better choice, at least in terms of the prevention of subsidence, than autogenous iliac bone graft for the treatment of subarticular defects associated with unstable tibial plateau fractures [485]. Furthermore, BoneSourceTM was found to be safe and effective when used to fill traumatic metaphyseal bone voids and appeared to be at least as good as autograft for treatment of these defects [486]. As this manuscript is intended to be read mainly by chemists and materials researchers, the biological, medical and clinical aspects of calcium orthophosphate cement applications have not been discussed in many details. For further biomedical details, the interested readers are referred to other papers and reviews [21, 25, 32, 33, 111, 391, 395, 477].

9. FUTURE DEVELOPMENTS

As calcium orthophosphate cements and concretes represent an intriguing group of new materials for bone augmentation and reconstruction, there is a great potential for further improvement of their properties, in which the ideal characteristics (Table 4) should be approached by manipulations with the chemical composition, powder particle size and distribution, as well as by means of various additives. Several commercial cement formulations have been already approved for a clinical application [111, 175, 397, 410]. New formulations of both apatite and brushite cements are expected to appear in the market soon. The forthcoming commercial formulations will need to be improved in order to take the advantage of a variety of possibilities offered by calcium orthophosphate cements. New formulations will include (i) injectable and open macroporous formulations to optimize their osteoconduction [240], (ii) formulations containing only one calcium orthophosphate (single-phase cement powders) [17] and (iii) drug-loaded and hormone-loaded cements for the treatment of bone diseases [25, 32, 33]. Obviously, the former two directions deal with both chemistry and material science, while the last direction is more related to tissue engineering and medicine.

Two innovative approaches of injectable cement formulations have been introduced relatively recently. The researches combined a water-reactive apatite cement such as a mixture of TTCP and DCPD powders with a nonaqueous but water-miscible liquid (*e.g.*, glycerol, polyethylene glycol) + a gelling agent (*e.g.*, hydroxypropylmethylcellulose, carboxymethylcellulose, chitosan) + a hardening accelerator (*e.g.*, tartaric acid, malic acid, malonic acid, citric acid or glycolic acid) to form a stable paste that can be directly injected into a bone defect [487-489]. In literature, this type of cement pastes is called “premixed calcium phosphate cements” (occasionally referred to as PCPC) in which the paste remains stable during storage and hardens only after placement into the defect. Setting occurs upon contact with body fluids or in a physiological solution and results in CDHA formation. This

approach eliminates the powder-liquid mixing stage during surgery and might improve the cement performance. Besides, it allows shortening the surgical time, as well as the risk of operator-induced error is considerably reduced.

The first formulation of premixed calcium orthophosphate cements had a setting time of longer than 1 h and a low mechanical strength [487]. Afterwards, an improved formulation has been developed; it exhibits a rapid setting when immersed in a physiological solution, yielding a hardened cement with a higher mechanical strength, approached the reported strengths of sintered porous HA implants and cancellous bone [488, 489]. Creation of premixed macroporous calcium orthophosphate cement scaffolds reinforced by slow-dissolving fibers (in other words, premixed macroporous concrete scaffolds) is the latest achievement of this approach [339]. Other researchers invented cements in the form of two injectable pastes that could be mixed together and injected at the time of implantation (with a static mixer incorporated in the injection cannula) [490]. Nevertheless, the latter approach is limited to acid-base cement formulations only [30].

To date, no study has reported on a possibility of the premixed brushite formulations at ambient temperatures. However, recently the researchers have discovered a way to overcome this problem at low temperatures [129]. Three different pre-mixed brushite cement formulations formed by freezing the cement pastes following combination of the powder and liquid components. When frozen and stored at -80°C or less, significant degradation in compression strength did not occur for the duration of the study (28 days). Interestingly, in the case of the brushite cement formed from the combination of β -TCP with 2 M H_3PO_4 solution, freezing the cement paste had the effect of increasing mean compressive strength fivefold (from 4 to 20 MPa), which was accompanied by a reduction in the setting rate of the cement. This strength improvement was attributed to a modification of crystal morphology and a reduction in damage caused to the cement matrix during manipulation [129].

A lack of macropores is a substantial disadvantage of many current formulations of calcium orthophosphate cements [237]. As a result, biodegradation takes place layer-by-layer on the surface, from outside to inside. To solve this problem, soluble particles, such as sugar [491], mannitol [336, 340, 492], NaCl [258, 259] and calcite [188], or resorbable fibers [340, 366-368] might be incorporated into the cement. After the cement is implanted, the particles are dissolved, leaving pores in the cement matrix; however, such pores are not always interconnected. Using a hydrophobic liquid instead of soluble particles could be an alternative. At the turn of the millennium, an open macroporous structure was obtained using a mixture of oil and a cement paste [493]; however, since then no research papers on this subject have been published. Besides, by means of surfactants, air bubbles might be created in the bulk of the cements [269]. Finally, addition of carbonates to the cement formulation is able to create pores [29, 46, 264, 265]. Unfortunately, the mechanical strength and porosity are conflicting requirements. As the porosity in calcium orthophosphate cements appears to be of paramount importance to achieve the excellent bioresorbability, other experimental approaches have to be developed [494].

Recently, a layered structure was designed by combining a macroporous layer of calcium orthophosphate cement with a strong fiber-reinforced calcium orthophosphate cement layer. The rationale for such construction was for the macroporous layer to accept tissue ingrowth, while the fiber-reinforced strong layer would provide the needed early-strength [495].

Table 4. Major advantages and disadvantages of the calcium orthophosphate cements [32, 33, 172].

Advantages	Disadvantages
1. Self-setting ability <i>in vivo</i>. 2. Good injectability that allows cement implantation by minimally invasive surgical techniques, which are less damageable than the traditional surgical techniques. 3. Good osteoconductivity and occasional osteoinductivity: the initial biological properties of the hardened cements are similar to those of CDHA or brushite. 4. Can be replaced by newly formed bone after a period of time (osteotransductivity). 5. Moldability: the perfect fit to the implant site, which assures good bone-material contact, even in geometrically complex defects. 6. Excellent biocompatibility and bioactivity. 7. No toxicity. 8. Low cost. 9. Ease of preparation and handling. 10. Setting at body temperature. 11. Form chemical bonds to the host bone. 12. Clinically safe materials in their powder components. 13. Can be used to deliver antibiotics, anti-inflammatory drugs, growth factors, morphogenic proteins, <i>etc.</i> at local sites, which are able to stimulate certain biological responses.*	1. Mechanical weakness: limited use due to potential collapse of material followed by soft tissue formation instead of bone formation (loaded areas). Until cements with adequate shear strength are available, most complex fractures that can be repaired with cement also will require metal supports. 2. Can be washed out from surgical defect if excess of blood. 3. Lack of macroporosity (especially interconnected pores), which prevents fast bone ingrowth and the cements degrade layer-by-layer from the outside to the inside only. 4. The <i>in vivo</i> biodegradation of many formulations is slower than the growth rate of a newly forming bone.

*Further studies are necessary.

In the case of calcium orthophosphate concretes, future studies could combine in one formulation porogens and biodegradable fibers of different shapes and dissolution rates to form after *in vivo* hardening calcium orthophosphate scaffolds with sustained strength. In such a system, one porogen quickly dissolves and creates macropores to start a bone ingrowth process, while the second type of fibers provides the required strength to the implant. After significant bone ingrowth into the initial pores increased the implant strength, the second set of fibers would then dissolve to create additional macropores for bone ingrowth [371]. Such complicated formulations have already been developed. For example, chitosan, sodium orthophosphate and hydroxypropylmethylcellulose were used to render calcium orthophosphate cement fast setting and resistant to washout, while absorbable fibers and mannitol porogen were incorporated for strength and macropores, respectively. Both the

strength and fracture resistance of this concrete were substantially increased and approached those values for sintered porous HA implants [496]. Turning on a bit of imagination, one might predict development of polymeric drugs [497], hormones, growth factors, *etc.* (*e.g.*, by either incorporation into or cross-linking with either water-soluble or bioresorbable polymers). Coupled with reinforcing biodegradable fibers and porogens, such types of “healing fibers” might be added to calcium orthophosphate concretes, which not only accelerate the remedial process, but also allow simultaneous improvement in both their strength and injectability.

Stability (insolubility) in normal physiological fluid environment and resorbability under acidic conditions produced by osteoclasts appears to be among the most important *in vivo* characteristics of modern calcium orthophosphate cements and concretes. For some clinical applications, such as cranioplasty, a relatively slow resorption and replacement by bone is quite acceptable, whereas in other applications, such as periodontal bone defects repair, sinus lift, *etc.*, the ability of the hardened cement to be replaced quickly by bone is crucial. Experimental results suggest that a number of parameters of calcium orthophosphate cements, such as the Ca/P ionic ratio, carbonate content, ionic substitution, crystallinity, *etc.* might affect the dissolution characteristics of the cements in slightly acidic solutions. This gives an opportunity to formulate cements, possessing different resorption rates, which is suited for different applications [117, 118].

The discovery of calcium orthophosphate cements and concretes has already opened up new perspectives in synthesis of bioceramic scaffolds, possessing sufficient mechanical properties [249, 250, 274, 336]. In the past, such scaffolds could only be manufactured by the sintering route at elevated temperatures. Therefore, until recently it was impossible to produce resorbable preset low-temperature hydrated 3D ceramics for various applications, *e.g.*, scaffolds and granules, from low-temperature calcium orthophosphate phases, such as ACP, DCPA, DCPD, OCP and CDHA. Now, using the appropriate techniques, open macroporous 3D scaffolds consisting of the aforementioned low-temperature phases (currently, excluding ACP and OCP) can be produced via a cementitious reaction [492, 498-501], thus dramatically widening the application of these calcium orthophosphates as biomaterials and bioceramics. This type of materials could be very promising for tissue engineering applications. Among them, CDHA is of a special interest due to its chemical similarity to bone material and a large specific surface area.

To conclude this part, one should stress, that the most promising direction of the future developments of calcium orthophosphate cements and concretes is obviously seen in their functionalization by incorporation or impregnation of various hormones, growth factors, drugs, other bioorganic compounds, as well as incorporation of living cells and other tiny biological objects [502-507]. The initial attempts have already been performed but without a great success yet. For example, researchers have already found that unset calcium orthophosphate cements might have toxic effects when placed on cell monolayers, while the set cements are biocompatible for the same type of cells (MC3T3-E1 osteoblast-like cells were tested). A gel encapsulation in alginate beads was found to be a possible solution to protect living cells for seeding into calcium orthophosphate cement pastes [508]. *In vitro* cytotoxic effect of a calcium orthophosphate cement based on α -TCP was also observed [509]. In light of these results, the encapsulation approach [255] could potentially be used to seed a patient's *ex vivo* expanded stem cells into a cement to create an osteoinductive bone

graft substitute that could be used to treat that patient. However, this becomes more related to tissue engineering and biology, rather than to chemistry and material science.

Finally, besides the aforementioned chemical, material and biomedical improvements of calcium orthophosphate cements and concretes, one should not forget on a better design of both the mixing equipment and delivery (injection) techniques. As an example, the interested readers are referred to a new cannula to ease cement injection during vertebroplasty [510]; however, this subject is beyond the scope of current review.

10. CONCLUSIONS

Thus, among the diverse range of bone replacing biomaterials, calcium orthophosphate cements and concretes undoubtedly represent a distinct group because they are relatively simple materials formed by combining a calcium orthophosphate mixture with an aqueous solution. However, they symbolize an important breakthrough in the field of bone repair biomaterials, since they offer the possibility of obtaining thermally unstable calcium orthophosphates in a monolithic form at room or body temperature by means of a cementation reaction. This particular fabrication technique implies that the cements are moldable and therefore can adapt easily to the bone cavity providing a good fixation and the optimum tissue-biomaterial contact, necessary for stimulating bone ingrowth into them and their subsequent osteotransduction [25].

Unfortunately, the perfect grafting material does not exist. Calcium orthophosphate cements and concretes are not an exception to this statement. While possessing excellent biological properties (osteoconduction and, occasionally, osteoinduction), adequate setting time, excellent moldability and the capability to deliver different bone-enhancing proteins/antibiotics at a local level, unfortunately, the material lacks adequate mechanical properties for applications other than non-loaded surgical sites (see Table 4 for other details). Nevertheless, even in its present state calcium orthophosphate cements appear to be suitable for a number of applications. They can be injected into osteoporotic bone to reinforce it or can be used to make granules and blocks out of low-temperature calcium orthophosphates. Several types of calcium orthophosphate cements are now on the market, while scaffolds made of low-temperature calcium orthophosphates are being tested. The use of slightly different chemical compositions and various dopants affects both the setting time and tensile strength that enables further improvements. In addition, new trials are conducted with the reinforced formulations and concretes, which represent additional attempts to improve the existing products.

It is anticipated that the use of calcium orthophosphate cements will enable a faster and more aggressive rehabilitation, as the strength of the cement makes it possible to allow full weight-bearing earlier than when bone graft is used. Although, preliminary clinical trials have already confirmed the great potential of this novel therapeutic product, calcium orthophosphate cements need to be improved further; in particular, their bioresorption needs to be accelerated as well as their injectability and mechanical properties need to get better. Besides, extra clinical studies are required to define the most appropriate indications and limitations of calcium orthophosphate cements for fracture repair.

In the author's humble opinion, mentioning the Prof. James M. Anderson's opinion on the history of biomaterials field would be the best way to conclude this review. According to Prof. Anderson, within 1950 – 1975 researchers studied bioMATERIALS, within 1975 – 2000 they studied BIOMATERIALS and since 2000 the time for BIOmaterials has been coming [511]. Here, the capital letters emphasize the major direction of the research efforts in the complex subject of biomaterials. As the history of calcium orthophosphate cements started only in 1983, the aforementioned periods were shifted along the time scale. Certainly, the bioMATERIALS-epoch for calcium orthophosphate cements is almost over (every possible combination of the cement formulation has been already tested), while the BIOmaterials-era (where cells are the key factor) either has not started yet or is just at the very beginning. Most likely, current state-of-the-art of calcium orthophosphate cements and concretes corresponds to BIOMATERIALS-phase with an approximately equal contribution of biological and materials directions. Therefore, still there is much room for versatile ideas and approaches.

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Chapter 3

HYDROGELS IN BIOLOGY AND MEDICINE

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ABSTRACT

The range of materials used for biomedical applications is very broad. This means that the demands on their properties are very diverse depending on various medical areas and applications. Moreover, it is often necessary to have available materials with the possibility to set the required parameters very precisely in very wide ranges. Because of the similar mechanical behaviour of hydrogels with that of living tissues and their good compatibility and ability of hydrogels to swell in water, the hydrogels are often used in biomedical applications.

Hydrogel polymers are natural or synthetic hydrophilic crosslinked polymers favourably interacting with water. The swelling degree (the water content in equilibrium-swollen gel) is a function of polymer hydrophilicity and the degree of crosslinking and has influence on a number of physical and chemical properties (e.g., refractive index, transport properties). In biomedical applications, the physical structure and morphology of the hydrogels is adjusted to the targeted performance and both homogenous and heterogeneous materials with porous, nanofiber, or nanoparticle structures are used.

One of the oldest and still widely used biomedical applications of hydrogels is the contact lens. Hydrogels are successfully applied as a variety of implants in surgery, ophthalmology, otorhinolaryngology, neurology, urology, gynaecology etc. In addition, hydrogels are used to cover wounds, burns, trophic defects, or as a two-dimensional supports for cultivation and potential transplantation of cells or as three-dimensional scaffolds for tissue engineering and cell therapy.

In some applications, particularly, in tissue engineering, the biodegradable materials (hydrogels) are used. The advantage is that after the fulfilment of their tasks (e.g., proliferation of cell culture), they disintegrate (hydrolytically or enzymatically) and, subsequently, they are eliminated from the organism. Controlled transport and release of drugs is a separate biomedical area.

Hydrogels for biomedical applications can be generally classified in different categories depending on their interaction with the living tissue. The hydrogels are used

either in direct contact with tissues (implants, injection needle, infusion sets, bandages, suture materials, carrier of drugs, cell or tissue culture) or in indirect contact with tissues (orthosis, medical devices, air filters, sanitary supplies, etc.).

The matter of this chapter is arranged in the following subchapters:

1. Introduction
2. Structure of hydrogels in relation to the formation conditions
3. Swelling and mechanical properties of hydrogels
4. Contact lenses
5. Intraocular lenses
6. Functional implants
7. Embolization of blood vessels
8. Wound-dressing
9. Conductive supports for contact electrodes
10. Hydrogels for tissue engineering

1. INTRODUCTION

No exact definition of the "Gel" or "Hydrogel" state exists. It is commonly accepted that the gel is a viscoelastic solid containing a certain amount of liquid. The viscoelastic characteristics vary in a certain range corresponding to the softness of the gel and certain shape reversibility when external stresses are applied (cf., e.g., [1]). The Encyclopedia of Polymer Science offers the following definition for *hydrogel* "*Hydrogels* are hydrophilic polymers that absorb water and are insoluble in water at physiologic temperature, pH, and ionic strength because of the presence of a three-dimensional network" [2]. Since we will be discussing here polymer gels we will be using the latter definitions but we have to pay attention to the nature of bonds by which three-dimensional network structure is achieved. As far as "hydrogels" concerns, these are "gels" which favorably interact with water and in which water is the predominant medium during hydrogel service.

The bonds making from a polymer the gel are either covalent (permanent) or physical (transient). The covalent bonds are strong and durable. The majority of them does not dissociate, they are rather split or transformed by chemical reactions. The range of relaxation time (durability) of bonds is very broad. It falls down from years for covalent bonds to seconds for entangled polymer solutions. Some physical gels exhibit the behavior of covalent gels although the isolated individual bonds making them physically crosslinked are weak (hydrophobic interactions, hydrogen bonds). However, they are formed cooperatively and are grouped in sequences of various lengths (crystallites, multiple helices, etc., cf., e.g., [3]). The difference between covalent and sequential physical bonds exists in their temperature response. The covalent bonds usually do not dissociate upon increasing temperature until they degrade at temperatures exceeding the boiling point of water. A relatively sharp dissociation or "melting" temperature is characteristic for physical hydrogels. This dissociation is manifested by transition of the gel to solution or an abrupt change in the degree of swelling (cf., e.g., [4]). For hydrogels, the sol-to-gel transition often occurs on lowering the temperature.

In this chapter, we will concentrate on covalent gels. In hydrogels, the physical interactions always play an important role.

2. STRUCTURE OF HYDROGELS IN RELATION TO THE FORMATION CONDITIONS

2.1. General Features of Network Build-Up

Formation of hydrogels from the starting materials (precursors) by chemical crosslinking as well as physical association has some common features [5], [6]. To form hydrogels, the precursor must have a certain affinity to water. First, the molecular weights of the forming reaction or association products increase. The weight-average (M_w) and higher averages of molecular weight increase much steeper than the number-average molecular weight (M_n), so that the polydispersity index characterized by the ratio M_w/M_n also increases. If the system gels at all, there always exists a point, the gel point, characterized by critical conversion of the functional groups (covalent systems) or degree of association (physical gels). Experimentally, the gel point is described, respectively, by critical reaction time and critical concentration. Just beyond the gel point, the gel fraction (fraction of polymer of „infinite” molecular weight) appears which for covalently crosslinked gels is insoluble in any good solvent. The structural changes also serve for determining the gel point - particularly the divergence of M_w or the conversion at which the gel fraction is extrapolated to zero. Also, some other physical methods indicate the occurrence of the gel point like independence of the loss angle tangent of frequency in dynamic mechanical experiments [7], changes in the structure of laser speckle [8], time-resolved light scattering [9] or microrheology in which reaching of the gel point is manifested by a sudden change of slope of the dependence of the mean-square displacement of markers on time [10]. For the network (gel) formation a difference in the composition of the sol and gel is characteristic. Especially the difference of the degree of conversion (or association degree) of functional groups in the sol and gel is large.

In the region beyond, the gel point is characterized by a continuous transformation of soluble molecules into gel by reaction of the functional groups of sol molecules with the groups of the gel and build-up of the internal structure of the gel. This gel build-up is associated with increasing connectivity of the gel units manifested by increasing number of paths called elastically active network chains (EANC) and decreasing number of dangling chains composed of units connected only by single paths of bonds to the gel. The dangling chains are not active in resisting stresses applied externally [5],[6]. Typical changes characterizing covalent network formation are shown in Figure 1.

The gel point (located in this example at about 20% conversion) can vary from close to 0% conversion of reactive groups (crosslinking of long primary chains) to a value close to 100% conversion depending mainly on the functionality of precursors and stoichiometric disbalance in systems based on alternating reactions. The dependence of M_w is always curved upwards. The increase of the gel fraction is initially steep and then slowly converges to 100 %, but it is not always so. Sometimes, a part of the system remains soluble despite the conversion reached 100 %. The dependence of concentration of elastically active network chains on conversion is initially curved upwards and may go over to a linear dependence. The

shift of the gel point depends on functionality distribution of the precursor, group reactivities, and especially on the reaction mechanism. These issues will be discussed in Section 2.3.

2.2. Physical Polymer Hydrogels

Physical gels can have manifold structures depending on the type of association and concentration. The degree of ordering can be low for randomly distributed associating groups – stickers – or high for system containing ordered sequences (block copolymers, peptide units) which give rise to micellar, cylindrical, or layered lamellar networks (gels). The latter systems are the domain of supramolecular chemistry. Another class of hydrogels is biohybrid gels in which the biopolymer motifs, usually oligopeptides or polypeptides, are chemically attached to the synthetic hydrophilic chains. The gels are formed by association of the motifs and are liquefied by motifs dissociation. The motifs associate to form superhelices or β -sheets. The association is highly specific and dependent on the exact sequence of amino acids in the motifs, which is utilized in molecular recognition

2.3. Inter- and Intramolecular Crosslinking

A common phenomenon which is often overlooked is the formation of cyclic structures by intramolecular reactions. Before the gel point, formation of a bond between two functional groups of one molecule is an intramolecular reaction. In contrast to intermolecular bonds, formation of an intramolecular bond does not contribute to the increase of molecular weight (Figure 2).

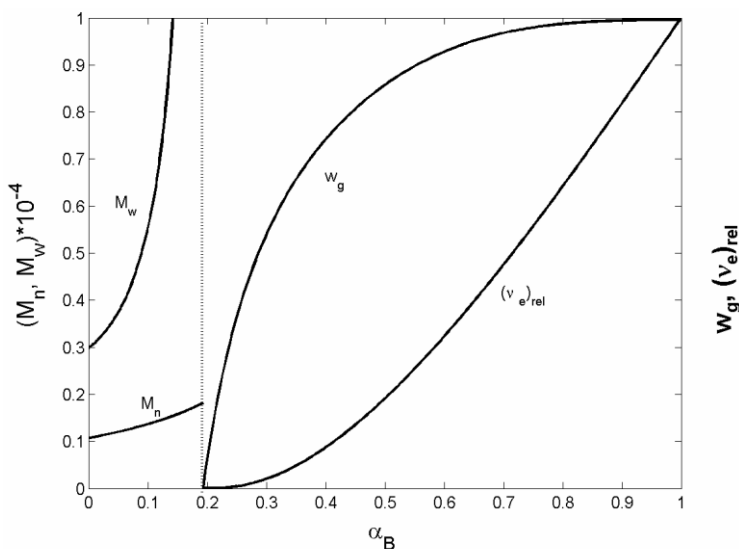


Figure 1. Development of molecular weight averages (M_n , M_w), gel fraction (w_g), and relative concentration of EANC's ($(v_e)_{rel}$) for a statistical copolymer of units with primary and secondary OH groups (2:1) and a non-functional monomer crosslinked with a triisocyanate; α_B is conversion of NCO groups [6].

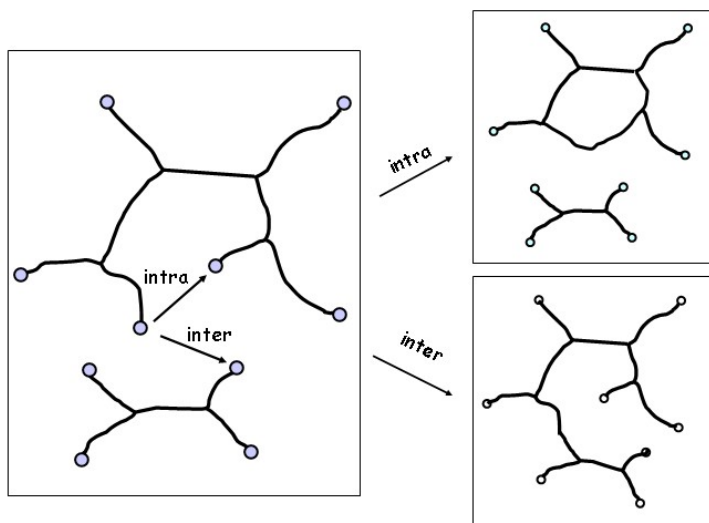


Figure 2. Inter- and intramolecular crosslinking before the gel point.

The molecular weight averages of a crosslinking system grow more slowly and the gel point is shifted to higher conversions [11] compared to the situation if all bonds were intermolecular. Beyond the gel point, some crosslinks are not effective in the increase of the concentration of EANCs. The extent of cyclization increases with dilution of the system. The shift of the gel point conversion in dependence on dilution during network formation characterizes the tendency of the given system to formation of cycles. Moreover, extrapolation of the dependence to the hypothetical infinite concentration of functional groups gives the ring-free value of the gel point conversion ($\alpha_g(\text{inter})$ in Figure 3). This is the value which can be relatively easily obtained theoretically.

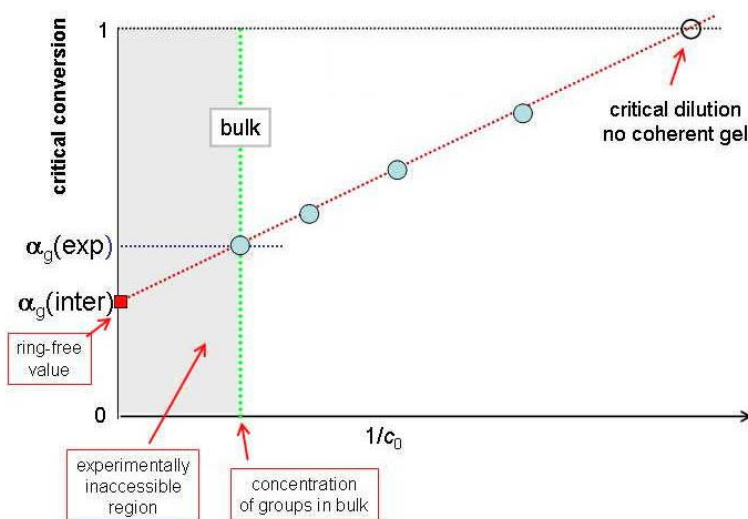


Figure 3. Determination of the effect of cyclization by measurement of the gel point conversion of functional groups, $\alpha_g(\text{exp})$, in dependence on the reciprocal initial concentration of functional groups $1/c_0$.

For physical gels, gelation is primarily dependent on temperature, especially for bonds formed cooperatively (helices, superhelices, β -sheets). However, in the temperature region where such bonds can exist, gelation is a function of concentration [11]. Because such critical concentrations are usually low, sometimes well below 1 wt.-%, a considerable fraction of bonds is intramolecular [12].

2.4. Ways of Formation of Polymer Hydrogels

This Chapter is not designed for listing various chemistries being used for synthesis and manufacture of hydrogels. It only discusses the significance of the mechanism of chemical network build-up for gelation and network structure. Basically, three ways of network formation exist differing in the mechanism of bond formation:

1. crosslinking of preformed polymer chains
2. chain (co)polymerization
3. step growth reactions

The boundaries are not sharp because preformed chains can be short or long and they may contain groups reacting stepwise or groups (e.g., C=C double bonds) reacting chainwise. The basic difference between step and chain reactions exists in the evolution of molecular weight distribution. In the case of step reactions, the functional group on any oligomer can react with any other corresponding group and the distribution develops from monomer to dimer, trimer, tetramers, etc. In the chain reactions, it is only the activated group that can react with a non-activated one. If the propagation step is relatively fast compared to initiation, the branched chains are initially “dissolved” in monomers. These differences in evolution of molecular weight distribution basically affect the position of the gel point and the onset of possible phase separation.

Comparing as an example crosslinking of a tetrafunctional tetraol with stoichiometric amount of a bifunctional diisocyanate and copolymerization of tetrafunctional glycol dimethacrylate with bifunctional methyl methacrylate, we find that the former, the step system, gels at around 60% conversion, whereas the latter, the chain system, can gel already at conversions of C=C bonds as low as 0.1 %. The corresponding values of the critical conversion (α_g) for the ideal ring-free case are given by relations

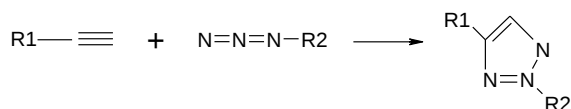
$$\begin{array}{ll} \alpha_g = [(f_H - 1)(f_I - 1)]^{-1/2} = 3^{-1/2} & \alpha_g = 1/x_4 P_w^0 \\ \text{step growth build-up} & \text{chain build-up} \end{array}$$

where f_H and f_I are numbers of functional groups of H-component (OH groups) and I-component (isocyanate groups), respectively, x_4 is the fraction of double bonds of the tetrafunctional monomer and P_w^0 is the weight average degree of polymerization of the polymerized sequences of bonds (“primary chains”).

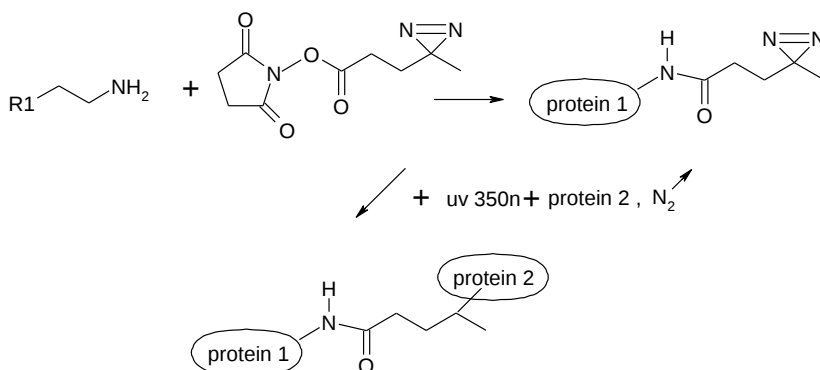
Polymer gels are prepared not only from monomers but frequently also from preformed precursors, such as telechelic polymers, functional stars, linear copolymer chains,

hyperbranched polymers, and various other prereacted systems [6]. Often, distributions exist not only in the molecular weight but also in the number of functional groups per molecule and distribution in chemical reactivities. These distributions can affect gel formation and gel structure considerably.

Recently, ways of formation of hydrogels were enriched by easily proceeding reactions in aqueous media, such as the click chemistry (cf., e.g., a compendium [13]).



Diazirine-based photo-reactive crosslinkers for crosslinking of amine functionalities including living cells [14] are interesting as well.



Hydrogels can be prepared in the absence of diluents and swollen in water after their preparation. Alternatively, preparation of hydrogels can occur in the presence of a diluent. The diluent is either water, or another additive which is then (possibly in several steps) exchanged for water. By varying the mechanism of network build-up and nature and amount of diluents, manifold morphologies of hydrogels of various chemical natures can be prepared (cf., e.g., [15]).

Traditional and widely used is free-radical polymerization and copolymerization of vinyl monomers containing a hydrophilic group, such as 2-hydroxyethyl methacrylate or acrylate, 2-hydroxyethoxyethyl methacrylate, glycerol monomethacrylate, acrylamide and methacrylamide, substituted acryl- and methacrylamides, monomers containing combination of hydroxyl and amide groups, carboxyl groups of acrylic or methacrylic acid. Some crosslinkers do not have sufficiently high affinity to water. Presence of a hydrophilic group like in glycerol dimethacrylate helps to solve this problem.

For hydrogels prepared by step growth reactions of alternating type, the effect of stoichiometric imbalance can be employed giving excess of hydrophilic groups in the network. Crosslinked polyamides from polyamines and polyacids, or crosslinked polyurethanes with excess of OH groups can serve as examples. The polyurethanes cannot be prepared in water or alcohols because of their reaction with the isocyanate group, but another solvent has to be used which is later exchanged for water. The crosslink density and hydrophilicity of the resulting network can be tuned by varying the off-stoichiometry, the

hydrophilicity of components and their functionality (including bifunctional and monofunctional components). To disentangle this maze of effects is a nice task for the branching theory. A prominent components bringing in hydrophilicity are oligomers or low-molecular-weight polymers of ethylene glycol.

Existing chains are in fact high-functionality precursors and can be crosslinked by step growth reactions like poly(vinyl alcohol) (PVA) with glyoxal, or chain reactions like PVA with grafted acrylic groups.

2.5. Special Features of Polymerization in Presence of Diluents. Phase Separation - Macrosyneresis vs. Microsyneresis

It sometimes happens, intentionally or undesirably, that the polymerizing system loses its thermodynamic stability and phase separates. This usually takes place if a diluent (poor or good solvent or other additive like a polymer) is present during polymerization. A two-phase system results – one phase is usually the (swollen) crosslinked polymer and the second phase is composed mainly of uncrosslinked additives. More complicated cases are not excluded – three phases in equilibrium, two crosslinked phases, etc.

The uncrosslinked phase can separate from the polymerizing system as a bulk (liquid) phase or the separated phases are interdispersed. These phase separation processes are called *macrosyneresis* and *microsyneresis*, respectively [6],[16]. The morphology of the gel depends on relative volumes of the separated phases (dispersion of diluent in the crosslinked matrix or dispersion of the crosslinked polymer in the liquid). The morphological features depend on several factors, such as interfacial tension, progress of polymerization at incipient phase separation – how far the system is from the gel point, how dense the network is, etc.

To understand the changes occurring during gel formation, one should examine the effect of swelling changes induced by such change in temperature by which the gel deswells. The result depends on crosslink density. If the crosslink density is relatively high the volume of the piece of gel decreases and the gel remains transparent (homogeneous). If the crosslink density is low, turbidity appears first and the volume of the gel very slowly decreases. The final result after long time (months) [3],[16] is the same as with macrosyneresis - two bulk phases (Figure 4).

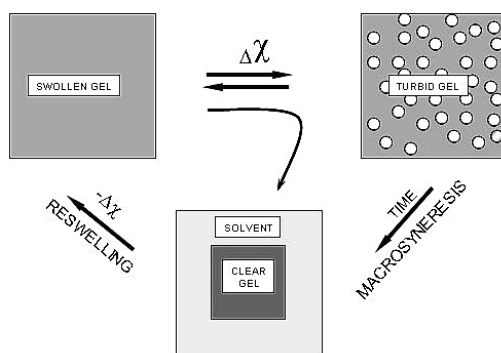


Figure 4. Changes induced in a gel by change of the interaction parameter χ (e.g., by change of temperature). Microsyneresis goes over to macrosyneresis.

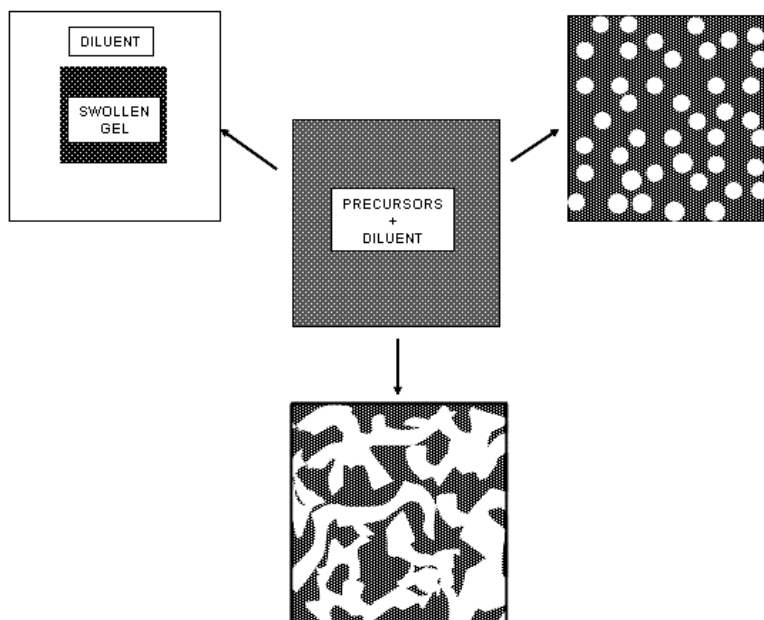


Figure 5. Phase separation occurring during network formation. Macrosyneresis and two forms of microsyneresis.

Microsyneresis causes first separation of the excess solvent in the form of “droplets” inside the gel and the droplets are under pressure of the locally deformed network. For low crosslink density, this pressure and the associated driving force for expulsion of the droplets are very low. In the case of reaction (polymerization) induced phase separation, the situation is similar. When phase separation occurs close to the gel point, where the effective crosslink density is low, phase separation always occurs by microsyneresis and the heterogeneous structure is fixed by proceeding crosslinking. The heterogeneous structure can never relax to two bulk phases. It is usually so when porous (macroporous) gels are prepared by adding diluents to the monomers. In some other cases, when the network is strong enough, macrosyneresis is the dominant phase separation mechanism [17] (Figure 5).

The reason for the phase separation is simple: the system cannot tolerate that much diluent it contains. The intolerance appears due to increasing crosslink density (concentration of EANCs, v_e). This is called v -syneresis. Deteriorating polymer-diluent interaction characterized by increasing polymer-solvent interaction parameter χ results in χ -syneresis. Sometimes, both factors – increasing v_e and increasing χ – are operative (cf., e.g., [17]). The effects of conversion degree at incipient phase separation and phase-volume ratios developed afterwards can be quantified using the swelling theory as will be shown in the Section 3.1.

Many hydrogel-forming systems are based on free-radical copolymerization in the presence of diluent. It should not be forgotten that the monomer(s) are not only parent materials for the hydrogel polymer but also a diluent. When an inert, non-polymerizing diluent is added we should consider the systems as a ternary one (disregarding the small amount of crosslinker) – network polymer-diluent 1 (monomer)-diluent 2 – or at least a pseudobinary system in which the diluent is a mixed solvent whose properties change continuously. For instance, HEMA with water form a cosolvent mixture for the polyHEMA

[18]. PolyHEMA gels swell better in the monomer than in water and the maximum degree of swelling is located at about 50 wt.-% water.

Figure 7 shows that the critical conversion at which phase separation starts decreases with increasing water content which also means that the pore volume as well as their connectivity increase. The cosolvency of water-HEMA mixture causes in fact a shift of the critical conversion to higher values compared to the situation when water and HEMA would interact specifically. These studies have further shown some other specific features of this mainly χ -driven phase separation: (a) the degree of swelling of the gel phase does not change much after phase separation has taken place; (b) in the region of dilutions exceeding the critical value but not too high, microsineresis can be accompanied by macrosineresis; (c) depending on the polymerization rate, reaction rate can exceed the rate of phase separation.

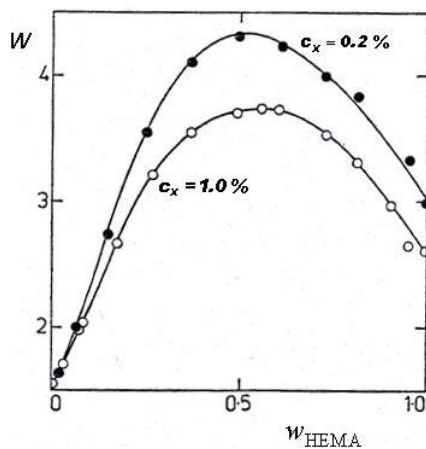


Figure 6. Equilibrium degree of swelling of polyHEMA gel in mixtures of HEMA with water. W = swollen weight/dry weight; c_x concentration of ethylene dimethacrylate crosslinker in wt.-%; w_{HEMA} weight fraction of HEMA in the swelling liquid; 50 °C; data of ref. [18].

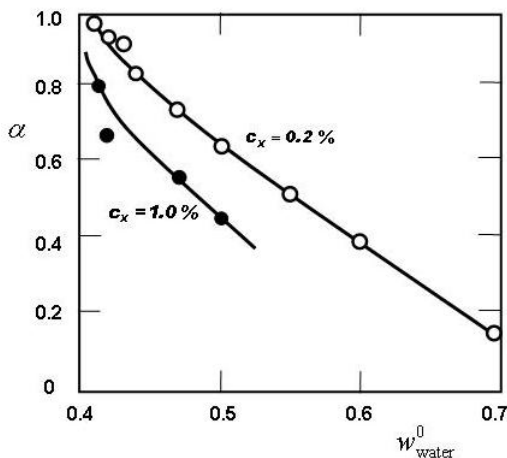


Figure 7. Conversion of double bonds of HEMA, α , in HEMA-water mixtures at incipient phase separation; w_{water}^0 is the initial weight fraction of water in HEMA-water mixtures; c_x concentration of ethylene dimethacrylate crosslinker in wt.-%; 60 °C; data of ref. [18].

Although the poor thermodynamic quality of water is the main driving force for phase separation, the concentration of crosslinker is also important. For swelling in water the increase of concentration of ethylene dimethacrylate (EDMA) from 0.2 to 1.0 wt.-% does not play any role, in the better cosolvent system of water with the monomer the difference in swelling degree is appreciable and because of that also the critical conversions differ (Figure 6 and Figure 7).

3. SWELLING AND MECHANICAL PROPERTIES OF HYDROGELS

3.1. Swelling Properties of Hydrogels

Swelling is the most characteristic property of a gel and swelling in water is characteristic for a hydrogel. Here, we will consider hydrogels that take up water (or other solvent) to a limited extent, i.e. they swell to equilibrium. The equilibrium degree of swelling at a given temperature and solvent vapor pressure is a characteristic property of a hydrogel. For several applications, also the swelling dynamics is important. However, the swelling and deswelling rates depend on sample geometry and on the initial state of the material (rubbery or glassy).

In this chapter, we will focus on swelling equilibria. In addition to weak interactions including hydrophobic interaction and hydrogen bonding, we will consider the effect of charged groups, conditions for three-phase equilibrium, and briefly ternary systems consisting of polymer-solvent1-solvent2. Based on the swelling thermodynamics, we will formulate the conditions for phase separation.

3.1.1. Equilibrium swelling

Swelling occurs because polymer segments have tendency to mix with solvent molecules. Mixing is associated with large gain in entropy. In the case of an uncrosslinked polymer, mixing of polymer with is affected by interactions between polymer segments and solvent molecules. At concentrations at which polymer coils overlap, a temporary, dynamic network can be formed having the properties of the gel in which the network chains are relaxed. If the interactions are changed, the physical gel can take up more solvent. It is eventually is transformed into a solution which can be further diluted.

In a covalently crosslinked network, the mixing tendency of polymer segments and solvent molecules is opposed by network connectivity and limited by chain elasticity. As a result of solvent uptake, the elastically active network chains are stretched and the resulting retractive force is counterbalanced the osmotic pressure.

In the thermodynamic treatment, the additivity of the Gibbs energies due to mixing and isotropic deformation of the network is usually assumed

$$\Delta G_{sw} = \Delta G_{mix} + \Delta G_{net} \quad (1)$$

For a binary systems, crosslinked polymer – solvent, the Flory-Huggins polymer solution theory and Flory, or Flory-Erman rubber elasticity theory give the following expression for ΔG_{sw} (cf., refs. [19]-[21])

$$\Delta G_{sw}/kT = n_1 \ln \phi_1 + n_1 \chi \phi_2 + n_e \left(A(\Lambda_x^2 + \Lambda_y^2 + \Lambda_z^2 - 3) - B \ln \Lambda_x \Lambda_y \Lambda_z \right) \quad (2)$$

where the deformation ratios Λ_i are related to isotropic reference state. For isotropic swelling, $\Lambda_x = \Lambda_y = \Lambda_z = \Lambda$

$$\Delta G_{sw}/kT = n_1 \ln \phi_1 + n_1 \chi \phi_2 + 3n_e (A\Lambda^2 - B \ln \Lambda) \quad (2a)$$

where k is Boltzmann constant, T temperature in Kelvin, ϕ_1 and ϕ_2 are volume fractions of solvent and polymer, respectively, n_1 and n_e are the number of solvent molecules and elastically active network chains (EANC), respectively, χ is the Flory-Huggins interaction parameter, A and B are factors in Flory-Erman junction-fluctuation rubber elasticity theory [21]. The deformation ratio for EANCs, Λ , is equal to

$$\Lambda = \phi_2^{-1/3} \phi_0^{1/3} \quad (2b)$$

where ϕ_0 is volume fraction of polymer components at network formation, $1 - \phi_0$ is the fraction of non-polymerizable diluent. The values of the factors A and B depend on how much interchain interactions affect fluctuation of crosslinks. Within the framework of this theory, there are two limits: $A = (f_e - 2)/f_e$, $B = 0$ for a phantom network with freely fluctuating crosslinks, and $A = 1$, $B = 2/f_e$ for suppressed fluctuation of crosslinks (f_e is the number of infinite paths issuing from an elastically active crosslink; in a perfect network it is equal to the chemical functionality).

From ΔG_{sw} , the change of the chemical potential of the solvent is obtained

$$\begin{aligned} \Delta \mu_1 / RT &= \ln a_1 = \ln(1 - \phi_2) + \phi_2 + \chi \phi_2^2 + V_1 v_e (A \phi_2^{1/3} \phi_0^{2/3} - B \phi_2) \\ a_1 &\approx p_1 / p_1^0 \end{aligned} \quad (3)$$

The swollen network is in equilibrium with pure solvent or with solvent vapor of partial pressure p_1 , p_1^0 being the vapour pressure of pure solvent. The activity of pure solvent is by definition $a_1 = 1$ and then the right-hand-side of Eq. (3) is equal to zero. If the gel is kept in contact with solvent vapors, it will swell less: the r.h.s of Eq. (3) will be less than zero. Thus knowing the structural parameters (χ , v_e , ϕ_0 , and for the given values of A and B), one can calculate the volume degree of swelling, expressed as $1/\phi_2$ equal to volume of swollen sample/volume of dry sample.

The swelling equilibria are schematically shown in Figure 8 as interdependence of the volume fraction of polymer and temperature for a system having lower (LCST) and upper (UCST) critical solution temperature, compared with an uncrosslinked (linear, monodisperse) polymer of increasing molecular weight.

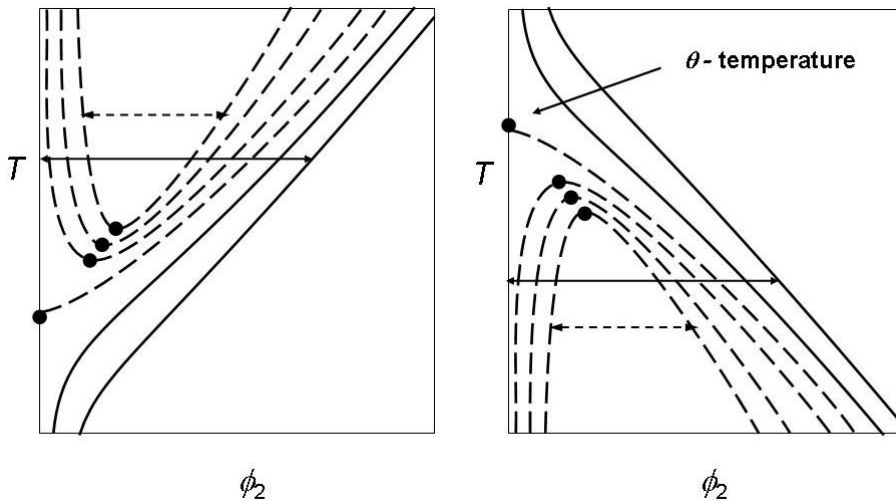


Figure 8. Partially miscible polymer solutions and swollen crosslinked polymers. Dashed curves - bimodals for a linear polymer of increasing molecular weight; the last curve for very high (infinite, M_{inf}) molecular weight; • critical points, the last for M_{inf} defines the θ -temperature. Horizontal double arrows define the composition of co-existing phases.

For uncrosslinked polymers, the coexisting phases have different polymer concentrations, the swollen gel is in equilibrium with pure solvent ($\phi_2 = 0$).

The degree of swelling of a crosslinked polymer is determined by

1. concentration of elastically active network chains, ν_e ,
2. polymer solvent interaction parameter χ (or g) and its concentration and temperature dependences,
3. memory parameter ϕ_0 characterizing dilution at network formation and thus the state of coiling of network chains in the dry network,
4. molar volume of the solvent, V_1 ,
5. functionality of the crosslink (average number of bonds with infinite continuation per elastically active crosslink, f_e),
6. ordering of the swelling liquid (liquid-crystalline solvents),
7. presence of macromolecular substances that lower solvent activity,
8. external strains, if applied.

Role of the interaction parameter χ

The goodness of solvents increases with decreasing χ . However, χ is usually not a constant but depends on temperature and polymer concentration. The interaction term should be then considered as an excess chemical potential. The temperature and concentration dependences of χ exist for many non-aqueous systems but are quite strong for aqueous gels. Several semiempirical have been offered but it is difficult to obtain the respective parameters independently and not just by curve fitting. The complicated dependence of χ is due to existence of different kinds of interacting sites on the polymer, hydrogen bonding, and iceberg structure of water affected by organic solutes (hydrophobic interactions).

The *temperature dependence* of swelling is positive for UCST systems and negative for LCST systems. For “organic” systems, usually but not always the UCST behavior prevails; aqueous systems usually show up LCST. However, the temperature dependence is sometimes more complicated, islands of partial miscibility are encountered. For instance, polyHEMA shows a shallow minimum at about 55 °C (Figure 9). Several functional forms for the dependence of χ on T have been offered in the literature. The most common is the dependence

$$\chi_T = a + \frac{b}{T} \quad (4)$$

corresponding to simple forms of entropic and enthalpic part of the interactions, sometimes the temperature dependence is more complicated (cf., e.g., [23],[24]) which leads to the presence of a logarithmic term

$$\chi_T = \frac{b_1}{T} + \ln \frac{c}{1+dT} \quad (5)$$

However, it is questionable whether the temperature dependence can be factored out as $\chi(T, \phi_2) = \chi_T(T)\chi_c(\phi_2)$, if a concentration dependence of χ exists. For a deeper discussion of the interaction function, see ref. [22].

Very important is the *concentration dependence* of χ .¹ The concentration dependence of χ is more frequent than a concentration independence. It can be generally expressed as power series of ϕ_2 for χ or g .

$$\chi_c = \frac{\chi_T}{1 - b\phi_2} = \chi_T(1 + b\phi_2 + b^2\phi_2^2 + b^3\phi_2^3 + \dots) \quad (6)$$

Often, the function is used quite successfully to express the combined concentration and temperature dependence. However, a strong concentration dependence of χ can give rise to so-called “off-zero critical concentration” [25],[26]. In this case, the critical concentration for a polymer of infinite molecular weight is not zero. Such dependence can induce a volume-phase transition (see below). The concentration dependence of χ is usually determined by measuring the degree of swelling as a function of crosslink density while the concentration of EANCs is determined independently. In this case, the concentration dependence of χ may also be affected by constraints to interaction imposed by the crosslinks (cf., e.g. ref. [27]). Therefore, it is better to determine the concentration dependence of χ by measuring the solvent uptake at various vapor pressures of the solvent.

¹ Equations (2) and (3) are selfconsistent only if χ is concentration independent, since the chemical potential is obtained from Gibbs energy by differentiation. Most frequently, the concentration dependence is determined by using eq. (3). Then, the concentration dependence of χ in eq. (2) is different in more recent literature [4], an interaction function $g(\phi_2)$ is used. The interrelations can be found in ref. [4].

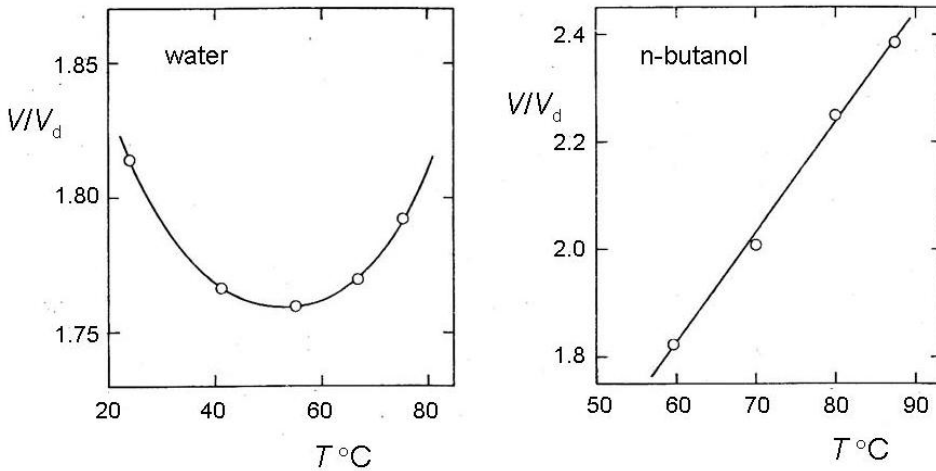


Figure 9. Temperature dependences of swelling of poly(2-hydroxyethyl methacrylate) gel crosslinked with 0.2 wt.-% (water) and 0.32 wt.-% (n-butanol) ethylene dimethacrylate and prepared in the presence of 40 wt.-% water. V/V_d - volume degree of swelling. Data of ref. [28].

In water, the swelling degree of polyHEMA passes through a shallow minimum at about 55 °C, whereas in more organic butanol the swelling degree increases with temperature almost linearly. However, many hydrophilic systems show up a decrease of swelling degree with increasing temperature. The popular polyNIPA (poly(N-isopropylacrylamide) can serve as example. The concentration dependence of the interaction parameter can be determined only in a very limited range of polymer concentrations because even uncrosslinked polyHEMA does not swell much. Therefore, a linear dependence is the only reasonable description but it may be used only for interpolation. The effect of hydrophobicity of the crosslinker is certainly contributing to this dependence. The dependence of χ for water swollen polyHEMA was found for the range of concentrations of ethylene dimethacrylate 0.1-2.7 wt.-% to satisfy the relation $\chi = 0.32 + 0.90\phi_2$ [29].

Effect of degree of crosslinking

It is very well known that the swelling degree decreases with increasing degree of crosslinking of the network. This can be understood inspecting Eq. (3): terms that are positive decrease the degree of swelling (increase ϕ_2). In hydrogels, the crosslinker units (like ethylene dimethacrylate) are hydrophobic and thus also the interaction parameter can increase with increasing crosslinking. To have a feeling of the effect of the degree of crosslinking, some calculated dependences are displayed in Figure 10 and Figure 11. The molar volume is arbitrary: it has been found that for treatment of aqueous systems by the Flory-Huggins theory, the best correlation was obtained if water was considered as trimer or tetramer of H₂O [22].

Effect of dilution during network formation

Very important is the effect of *dilution at network formation* characterized by ϕ_0 . Progressive dilution increases the degree of swelling. The purely diluting effect is manifest by a change of the chain conformation: the network chains are relaxed at the state of preparation

and they become supercoiled in the dry state as a result of shrinkage. Along with the effect on network chains conformation, dilution has some additional effects that tend to increase the degree of swelling: (a) dilution diminishes the interchain interactions and number of entanglements and (b) promotes closing of elastically inactive loops. Attainment of a certain value of ϕ_0 is necessary for the onset of liquid-liquid and liquid-gel phase separation during network formation and facilitates volume phase transition. Increasing *molar volume* of the swelling liquid, V_1 , makes the second term in Eq. (3) more positive and the gel swells less. The molar volume of solvent is an important factor particularly in achieving phase separation and manufacture of porous structures. Polymeric additives are much more efficient than their low-molecular-weight analogues.

A similar dependence illustrates the effect of dilution (Figure 11).

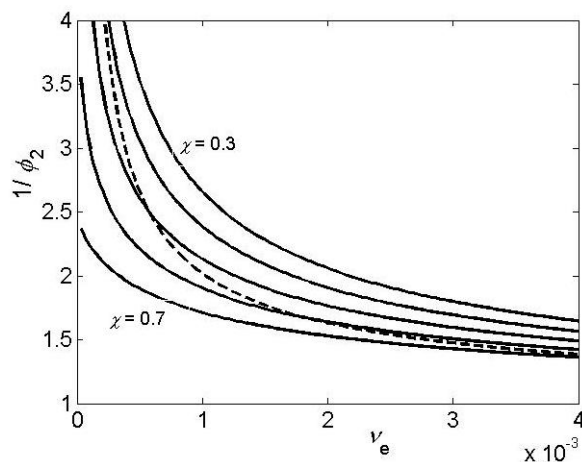


Figure 10. Dependence of volume degree of swelling according to Eq. (3) on the concentration of EANCs (mol/cm^3). Molar volume of solvent $V_1 = 100 \text{ cm}^3$, $\phi_0 = 1$. Full curves are calculated for concentration independent $\chi = 0.3, 0.4, 0.5, 0.6, 0.7$; the dashed curve calculated for the concentration dependence $\chi = 0.4 + 0.7\phi_2$.

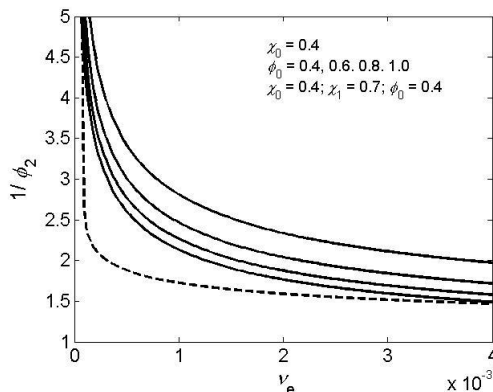


Figure 11. Dependence of volume degree of swelling according to Eq. (3) on the concentration of EANCs (mol/cm^3). Molar volume of solvent $V_1 = 100 \text{ cm}^3$, $\phi_0 = 1, 0.8, 0.6, 0.4$ from bottom to the top. Full curves are calculated for concentration independent $\chi = 0.4$; the dashed curve calculated for the concentration dependence $\chi = 0.4 + 0.7\phi_2$ and $\phi_0 = 0.4$.

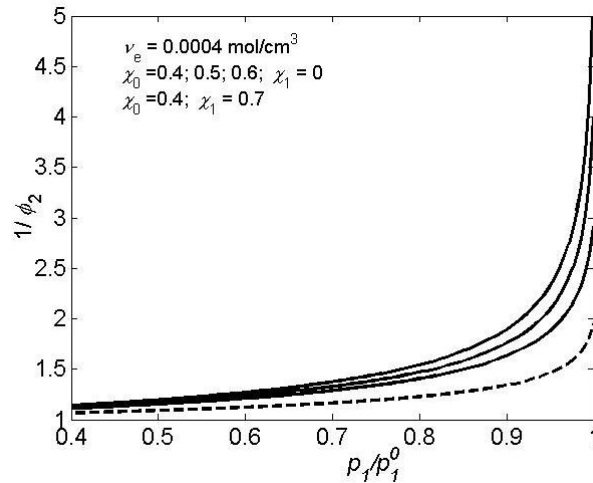


Figure 12. Dependence of the volume degree of swelling of the gel on relative vapor pressure of the solvent; concentration of EANCs 0.0004 mol/cm^3 , $\phi_0 = 0.6$; different values of concentration independent interaction parameter (full lines) are indicated. Dashed curve corresponds to the concentration dependent interaction parameter $\chi = 0.4 + 0.7\phi_2$.

Swelling in Solvent vapors

Determination of swelling of gels and hydrogels in solvent vapors is experimentally not so easy as swelling in liquid solvents but it is important scientifically as well as practically. For instance, hydrogel contact lenses during their service can be exposed to a relatively dry environment. The surface is then less swollen and even the transition of the soft gel to glass can set in. This transition will cause a drastic reduction of diffusion rate for the solutes and oxygen. Swelling Equation (3) predicts the effect of reduced vapor pressure - the solvent activity a_1 is equal to relative vapor pressure. Figure 12 shows the calculated effect of reducing the vapor pressure.

Characteristic for all these dependences is the sharp increase of the degree of swelling when the vapor pressure approaches saturation. This steep dependence makes the determination of the concentration dependence of the interaction parameter χ difficult. That the gels can approach the glassy state by decreasing the solvent content is another important fact. This rubber-glass transition can cause locking-in of some residual solvent in the glass. This residual amount (several %) is difficult to remove unless the polymer is transferred into the rubbery state by heating.

Swelling in water vapors was studied experimentally using the McBain quartz balances [30],[31] and also in conjunction with glass transition [32]. To avoid problems with water vapor condensation, the measurement was carried out up to $p_1 / p_1^0 \approx 0.9$. The measurement confirmed the hyperbolic shape of the theoretical prediction. For polyHEMA, the glass transition temperature of 25°C was found for about 10 wt.-% water. It can be expected that the main transition region extends to about 20 wt.-% water in polyHEMA. The sorption data were treated using the Flory-Huggins solution theory (Eq. (2)). As expected, the χ -values are high exceeding 1, but they rather decrease with increasing polymer concentration. This can be explained by locked-in excess free volume. Using the Lundberg clustering function, it comes

out that in these gels water has a strong tendency to form clusters. When the Flory-Huggins approach is used, clustering is reflected in a strong concentration dependence of the interaction parameter.

Effect of charged groups

Presence of charged groups in the polymer gel is an efficient tool for increasing the degree of swelling. Ionized or polyelectrolyte networks carry covalently attached ionized or ionizable groups (e.g., $-\text{COOH}$, $-\text{SO}_3\text{H}$, $-\text{N}(\text{Alk})_2$, $-\text{N}(\text{Alk})_3\text{OH}$). The degree of swelling of such networks can be very high. Some of them are strong acids or bases and are highly ionized. Some other has to be neutralized to function as fixed ions. Because of condition of electroneutrality, the charge of the ion fixed to the network is counterbalanced by a mobile ion of opposite sign (counter ion) (e.g., $-\text{COO}^- \text{X}^+$, or $(\text{Alk})_3\text{N}^+ \text{Y}^-$). The ions X^+ or Y^- can be exchanged for another ion. Such swollen networks are used as ion exchangers (cation exchanger and anion exchanger) for water treatment and other applications. Hydrogels carrying ionizable groups often exhibit volume phase transition (see below). The reason for a high swelling capacity of polyelectrolyte networks is the hydration of ions, especially of the counterions. In theoretical description of swelling not only mixing of network chain segments with solvent molecules and the elastic response of the network but also the effect of charges must be taken into account. The effect of charges is twofold: the hydration of counterions the concentration of which is controlled by Donnan equilibrium, and repulsive electrostatic interactions of fixed charges that contribute to chain extension. There exist several models to describe equilibrium swelling of polyelectrolyte networks. Usually, the additivity of Gibbs energies is assumed [4],[23]-[34]

$$\Delta G_{\text{sw}} = \Delta G_{\text{mix}} + \Delta G_{\text{net}} + \Delta G_{\text{ion}} + \Delta G_{\text{elst}} \quad (7)$$

ΔG_{mix} should include all non-electrostatic interaction, ΔG_{net} should respect the chain extension limit of chains due to high degrees of swelling encountered for polyelectrolyte gels (Langevin function or its expansion instead of Gaussian function). Of two last terms, $\Delta G_{\text{ion}} \propto \sum_j (c_j^{\text{gel}} - c_j^{\text{ext}})$ (the summation extends over all mobile ions) is more important than

ΔG_{elst} - the electrostatic repulsion of fixed charges). In equilibrium with external salt solution, the gel phase may also contain co-ions. Addition of co-ion causes screening of the electrostatic field produced by fixed charges and decreases the degree of swelling. To get an impression about the magnitude of the effect of charges the leading term of the polyelectrolyte effect - the Donnan effect - will be added to Eq. (3) and absence of added salt will be assumed. This modification is based on the extended model of Katchalski and Lifson [33],[34]. Several variants of theoretical approach to charged networks can be found in the monograph on the phase volume transition [4]. Using the variant of ref. [33], Eq. (3) is amended by the Donnan term

$$\Delta \mu_1 / RT = \ln a_1 = \ln(1 - \phi_2) + \phi_2 + \chi \phi_2^2 + V_1 v_e (A \phi_2^{1/3} \phi_0^{2/3} - B \phi_2) - i \rho \phi_2 V_1 / M_0 \quad (8)$$

where i is degree of ionization (from 0 to 1), ρ is the density of the polymer, M_0 the molecular weight of unit carrying one charge. This equation is valid for not too large degrees of ionization. Figure 13 demonstrate the effect of degree of ionization on swelling.

The results of Figure 12 show that ionization of only 50 % groups of a network of a 1:1 copolymer of a monomer bearing ionizable groups with an inert monomer and having low crosslink density can increase the degree of swelling ten times. However, for such a large expansion one should consider using the finite chain extensibility model as described in refs. [33] or [35].

Swelling transitions - two crosslinked phases

Analysis of Eqs. (2) and (3) has shown that a situation is possible, at which three phases can coexist: pure solvent in equilibrium with two crosslinked phases of different degree of swelling. By using Gibbs-Duhem relation, the chemical potential of polymer per equivalent segment $m = V_2/V_1$ is obtained in the form

$$\Delta\mu_2 / mRT = -\phi_2 + \chi\phi_1^2 + V_1V_e[A\phi_0^{2/3}(\phi_2^{-2/3}/2 + \phi_2^{1/3} - 3/2) + B(\ln\phi_2 + \phi_1)] \quad (9)$$

In equilibrium, the chemical potentials of components in either phase are equal: $\mu'_1 = \mu''_1 = \mu'''_1$ and $\mu'_2 = \mu''_2$. Three phases can coexist only in a certain range of parameters of the swelling equation.

For such systems, the calculated dependence of the chemical potential on ϕ_2 exhibits two maxima and the condition for phase equilibrium, equality of chemical potentials of a component in each phase, gave a real solution in the range of volume fractions $\phi_2 < 0,1 >$ [36]. Since for simple unionized networks it was difficult to achieve experimentally the proper combination of parameters (sufficiently high v_e at sufficiently high dilution), the experimental discovery of these transitions, characterized by a *jump in the degree of swelling*, was made 10 years later on hydrogels carrying *ionized groups* [37]. Also, it was found experimentally [4] and derived theoretically [25],[26] that nonionized systems with a complex concentration dependence of the interaction parameter (systems exhibiting the “off-zero critical concentration”) exhibit this transition. The best known of these systems is poly(n-isopropylacrylamide)-water [38] studied in hundreds of papers.

The volume phase transition can be induced by a number of stimuli, such as change in temperature, degree of ionization (pH), addition of co-ions, change in solvent composition, irradiation, application of electric or magnetic fields. The phenomenon of phase transition is widely utilized in controlled drug delivery. For instance, a gel conjugate when administered *per os* must pass through hostile environment before it reaches its target characterized by a certain value of pH. Then, a sudden expansion in volume exposes the gel interior to agents that split off the drug and rapid delivery of the drug is guaranteed. Other applications include concentration of dilute solutions of higher-molecular-mass, for instance, in technology of pharmaceuticals; for some superabsorbent gels, and various control devices where a fast collapse or expansion of gel-like materials is essential. There exist a number of monographs and reviews on this subject, such as refs. [4] and [39].

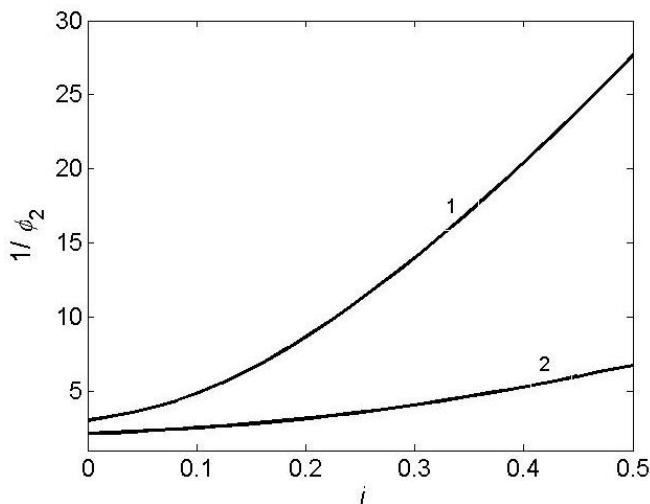


Figure 13. Effect of the degree of ionization, i , on volume degree of swelling; $\chi = 0.5$; $V_1 = 100$, $M_0 = 200$; curve 1 - $\nu_e = 0.0005$, $\phi_0 = 0.6$; curve 2 - $\nu_e = 0.001$, $\phi_0 = 1.0$.

Multicomponent systems

In numerous systems, more components than a crosslinked polymer and one diluent exist. The thermodynamic quality of the mixed solvent can vary linearly with composition, but often passes through a maximum (cosolvency) or minimum (anti-cosolvency). Cosolvency is encountered more frequently. Anti-cosolvency has been found less frequently when the two diluents strongly interact and tend to form a complex. As was already pointed out, all gels formed from a monomer by free-radical polymerization in the presence of an inert diluent are to be considered as ternary or better pseudoternary ones because the monomer has also a function of diluent. The possible situations for ternary systems are shown in Figure 14 using the triangle diagrams.

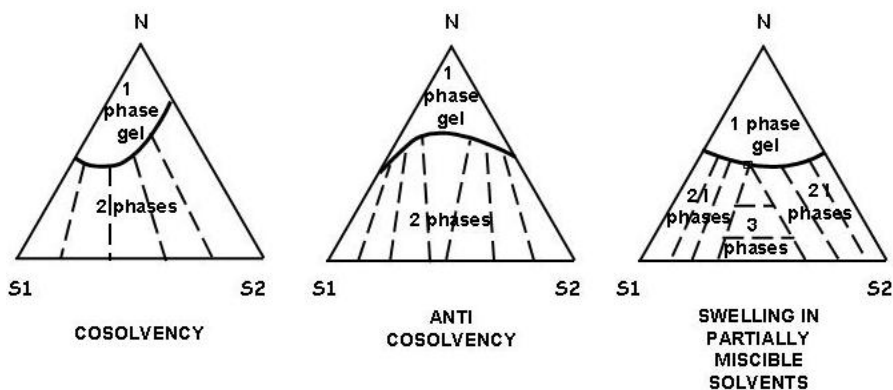


Figure 14. Swelling of a gel in binary solvent; N is network (crosslinked polymer), S1 and S2 are solvent 1 and solvent 2, respectively. The full curve delimits the single-phase region; the dashed lines are tie lines showing composition of coexisting phases.

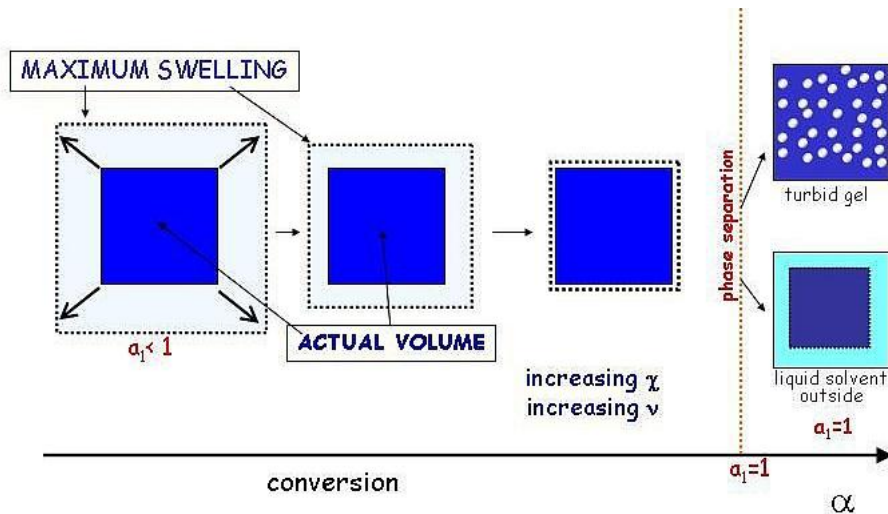


Figure 15. Changes occurring during network formation in the presence of a diluent; a_1 is the activity of diluent. For more detailed explanation see text.

The diagrams reflect the fact that a crosslinked polymer absorbs only a limited amount of liquids. The first two triangles show systems with maximum and minimum in compositional dependence of the degree of swelling. The third diagram shows the situation when S1 and S2 are only partially miscible and there exists a certain region of coexistence of three phases. One can expect that, for instance, the system crosslinked polyethylene glycol – water – benzene may show up a similar behavior. More frequent are systems *network polymer – solvent – nonsolvent* with a very asymmetric swelling curve.

Theoretically, the description of multicomponent systems is complicated [25]. The simplest description explained in detail the monograph by Tompa [40] is based on pairwise interactions quantified through three interaction parameters χ_{12} , χ_{13} , χ_{23} . Very often a ternary interaction parameter χ_{123} is necessary, but even then the interaction parameter for mixtures of two liquids may vary with composition. Some other discussion pertinent to polyHEMA-HEMA-water system can be found in ref. [41].

Condition for phase separation during network formation

In Chapter 2, we have already discussed microsineresis and macrosineresis which may take place during formation of a gel. It is clear and widely accepted that thermodynamic instability of the system is the reason for phase separation. Intuitively, one can expect that there has to be some relation between the swelling equation and loss of thermodynamic stability because the reacting system is a swollen network in equilibrium with solvent vapor. We will further restrict ourselves to a binary system network polymer – single solvent. We will inspect the swelling Equation (3). The situation can be visualized by scheme in Figure 15.

Dark blue squares show the actual volume of the polymerizing systems (gel swollen not to maximum) which is constant until phase separation sets in; the activity of solvent $a_1 < 1$. The light gray-blue region shows the hypothetical volume of gel if the reaction were stopped and the gel were brought into contact with excess of solvent. As the reaction conversion

increases, the solvent activity in the sample also increases until it reaches the value 1, which corresponds to pure solvent. At this point, phase separation starts. Beyond this point, a_1 remains equal to 1 (it cannot grow over this value) and increasing crosslinking causes separation of more and more diluent. In terms of ϕ_2 and ϕ_0 , the incipience of phase separation is defined by [42]

$$\phi_2 = \phi_0 \quad (10)$$

By inserting the condition (10) into the swelling Equation (3) the critical amount of diluent is defined

$$\begin{aligned} \ln(1 - \phi_0) + \phi_0 + \chi\phi_0^2 + V_1\nu_e\phi_0(A - B) &= 0 \\ (A - B)_{\text{ph}} &= \frac{f_e - 2}{f_e}; \quad (A - B)_{\text{af}} = 1 - \frac{2}{f_e} = \frac{f_e - 2}{f_e} \end{aligned} \quad (11)$$

It is interesting to note that the two limits of the Flory-Erman junction-fluctuation theory, one gets the same result $-(f_e - 2)/f_e$ (f_e is the average number of infinite paths issuing from an elastically active branch point), which close to the gel point give the value 1/3, because very close to the gel point $f_e = 3$ irrespective of the chemical functionality of the crosslink [43].

When applying the condition (11), one has to realize that changes of ν_e and possibly χ are the driving force for reaching phase separation. Thus, in applying Eq. (11), ν_e is to be taken as a function of conversion and what is actually found is the critical conversion at the given dilution.

3.1.2. Swelling under confined conditions

In many applications, especially in medicine, gels cannot always swell freely because they are geometrically confined by the space available or by developing objects (cells, tissues). The gel objects can be confined in one dimension (swelling between two plates), in two dimensions (gel confined in a cylinder), or three dimensions (gel enclosed in a cavity). The confining wall can be rigid, or elastic yielding somewhat to the swelling pressure. Questions arise: How much does such confined gel swell? How much is swelling anisotropic? If confined, what are the forces generated by the gel and acting against the confining wall?

To predict such effect, we can employ the change of the Gibbs energy (Eq. (2)) defining the deformations along the principal axes x , y , z and find interrelations between the deformation ratios using bounds such as constancy of the dry volume. The anisotropic version of Eq. (3) can serve for calculation of the degree of swelling under constraint. Alternatively, the forces acting on the constraining walls can be calculated by applying the relation between the force, F_i and change in the Helmholtz energy ΔA_{sw} (for atmospheric pressure $\Delta A_{\text{sw}} \approx \Delta G_{\text{sw}}$)

$$F_i = \frac{\partial \Delta A_{\text{sw}}}{\partial L_i} \quad (12)$$

where L_i is the sample length along the coordinate axis i . Confinement during network formation or arising during network formation can affect phase separation and other processes (distribution of chain extensions). In the past, such approach demonstrated the effect of constraint by adhesion on vapor pressure of a solvent evaporating from a coating film during its drying [44].

3.1.3. Other Important Issues

There are several other important issues related to swelling not discussed here. Certainly, it is the dynamics of swelling depending on whether swelling is connected with glass transition or not, and dependences on sample geometry. Also issues of hysteresis in transitions rubbery gel $\rightarrow$ glassy gel and glassy gel $\rightarrow$ rubbery gel and the associated excess free volume locked-in in the glass are interesting. Swelling of heterogeneous structures and determination of the swelling degree of the polymer matrix and pore volume in the swollen state are important issues as well. We believe that studies of solvent sorption as a function of vapor pressure can throw more light on this problem.

3.2. Mechanical Properties of Hydrogels

The key mechanical property of hydrogels (and polymer gels) is their equilibrium modulus of elasticity determined in the swollen state. The modulus is a measure of the mechanical resistance of a sample against the external mechanical load. Commonly, in polymer science, the modulus is expressed as a ratio between force acting onto a sample unit area, the stress, and the strain which is the deformation of the sample. The rubbery materials deform under loads reversibly to large deformations that span typically to hundreds of percent of elongation, and their recovery when the loading stops is very fast, instant by the theory. The swollen hydrogels behave to some extent as rubbers, they deform reversibly in a certain range although they rupture usually at much lower deformations compare to weakly crosslinked rubbers. The theory of rubber elasticity establishes the relation between an important structural parameter of the gel macromolecular structure: between the concentration of elastically active network chains and the mechanical response, that is the equilibrium modulus of elasticity. The fundamental thermodynamic concept for this relation was laid in the early days of polymer science by P.J. Flory [19]. The theory of rubber elasticity was being further developed by Mark and Erman [45].

The relationship between stress and deformation of an elastic network reads:

$$\frac{F}{A_{0S}} = G_{sw} (\lambda - \lambda^{-2}) \quad (13)$$

where F is force applied on a sample measured for instance by a tensile instrument, A_{0S} is the cross-sectional area of a non-deformed swollen sample (before the measurement), G_{sw} is the equilibrium shear modulus and λ is the deformation expressed as L/L_0 . L_0 is initial length (before deformation) and L is length of the deformed sample.

From the value of G_{sw} , obtained from the stress-strain measurement by Eq. (13), the so called crosslink density (ν_e) can be calculated using a theoretical equation for a swollen network in equilibrium:

$$\nu_e = G_{sw} / [RTA_f(\phi_2^0)^{2/3}(\phi_2^{1/3})] \quad (14)$$

where R is universal gas constant, T is absolute temperature (K), A_f is a dimensionless constant that characterizes the functionality of a crosslink. The ϕ_2^0 term is a volume fraction of macromolecular network at the network formation ($\phi_1^0 = 1 - \phi_2^0$, where ϕ_1^0 is the volume fraction of diluent present in gelling system). The ϕ_2^0 term called also “memory term” characterizes a state of macromolecular chains at the condition of network formation and is particularly significant for hydrogels as they are often prepared in diluted systems. The variable ϕ_2 is the volume fraction of macromolecular network in the swollen system macromolecular network–solvent in equilibrium and must be known for the given system for the same swelling solvent as at which the mechanical test is performed. To obtain ν_e in common units moles per cm^3 from the Eq. (14), one can for instance use the units: megapascals for G_{sw} and a factor of 10^6 in the denominator.

Instead of the not exact term “crosslink density” rather a well defined expression “concentration of elastically active network chains, EANC” should be used and understood. The language here grasps the essence of the thermodynamic relation; the equilibrium modulus in rubbery state is proportional to the number of molecular connections between the crosslinks per unit volume. The Eq. (14) gives the number of EANC per unit volume of a dry network which is a rational reference state at which the comparison of various systems should make sense. It is possible to derive from the same assumptions a relation for the number of EANC per volume of the swollen network (only the exponents will differ).

The constant A_f , so called front factor, falls into a range $<0.5 - 1>$ and it quantifies the effect of the average functionality of crosslinks on the equilibrium deformation behavior. In the so called affine network model, where the macroscopic deformation represents a sum of local microscopic deformations, the A_f value is equal to 1. In the so called phantom type model network the crosslinks are considered as fluctuating volume elements on a certain length scale, the value of A_f is defined as $(f_{ave}-2)/f_{ave}$ where f_{ave} is an average crosslink functionality. Because the minimum number of network chains extending from a crosslink is three to form a three-dimensional infinite network, the lower limit of the range for A_f is 0.5. To decide on which type of network should be considered to interpret given data, either affine or phantom, is not always a quick task and it may demand additional experiments or literature search on the properties of relevant systems. There is a vast literature available containing measured characteristics for many types of polymer networks and gels. In practice, the limit A_f value of 1 will serve well for an evaluation of a majority of covalently crosslinked swollen hydrogel samples. The comprehensive explanation of the theory of rubber elasticity can be found in the excellent books by Mark and Erman [21],[45].

Sometimes a misleading consideration about the molecular weight of network chains between crosslinks appears in the literature and textbooks on polymer science. An oversimplified relation $M_c = RT\rho/G$ is sometimes applied where M_c is meant to represent the average molecular weight of chains between crosslinks. This relation would be valid only for an ideal (dry, not swollen) network where all the chains are perfect and no dangling chains or

inactive loops and cycles appear. In real networks, the connection between crosslinks can have various lengths, branching, or they can bear dangling chains of different sizes on them. There can be unanchored chains pointing out from a crosslink. The expression in such instances is of course not valid. Therefore the structural characteristic parameter considered from equilibrium modulus measurement should always be the number of EANCs in unit volume (of dry network).

It should be noted that to get correct values of the equilibrium modulus, the measurement should not exceed the so called linear viscoelastic region that is a region where the relation between stress and strain is linear.

The equilibrium modulus can be measured either as a strain responding to introduced stress or vice versa, as a stress responding to defined strain. The equilibrium, i.e. the time independent values of the responses should be always sought for obtaining the structural parameters of the macromolecular network correctly. Relaxation or creep measurements are good choices.

There are several possible ways of the geometrical arrangement of the modulus measurement. It can be measured in tensile mode by applying force in the direction of the long dimension of a bar-shaped sample (Figure 16). The practical advantage of this way is the possibility of higher force response at very low deformation (high sensitivity) and well defined geometry of a sample. The sample held in clamps can also be loaded in torsion that means that usually one clamp rotates and thus deforms the sample. However clamping a soft hydrogel may be the bottle neck of the method even if a testing device of sufficiently high sensitivity (for soft matter) is found. The swollen sample can easily be drawn out from the clamps or can burst in the clamped part. The very soft samples will sag.

Such technical issues may be partially solved by use of torsion test geometry (Figure 17). An environmental chamber with temperature control and solvent excess into which the measuring geometry with the fixed sample is immersed during the test should be always applied.

There have been successful apparatuses contrived during the golden age of polymer physical science in the third quarter of twentieth century, in the predigital era. For example Cluff et al [46] constructed a simple compression modulus measuring device using a micrometer gauge and a series of weights. The working part of the apparatus, the two parallel plates of a plunger with the fixed pellet sample, could be immersed in a solvent during the measurement to prevent evaporation. The advantage of this design was its simplicity and a high precision at the same time and yet the device is quite inexpensive. In many laboratories who deal with hydrogel characterization such handy apparatus is used even today and its use is fully justified.

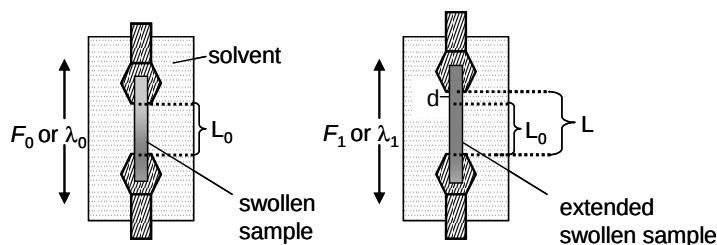


Figure 16. Tensile arrangement of the stress-strain measurement of a swollen hydrogel sample.

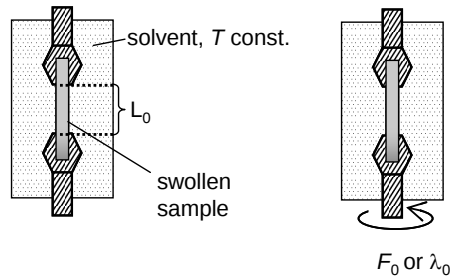


Figure 17. Stress-strain measurement in torsion. The torsion angle is very small, typically lower than one degree.

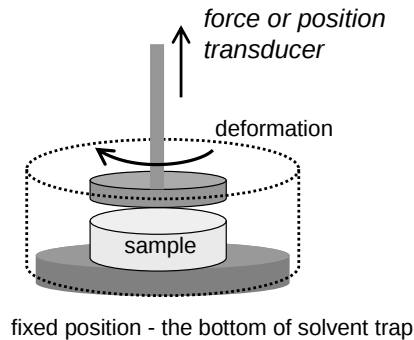


Figure 18. The scheme of shear oscillatory rheometer measuring geometry.

More sophisticated testing machines appeared in the early nineties. Their output became digital and the devices such as tensile modulus machine or various types of rheometers became computer-instrumented.

Nowadays, the controlling software and the hardware of fine multiple task machines for viscoelasticity characterization are inseparable elements. The common laboratory device suitable for hydrogel characterization is an oscillatory rheometer. Various measuring geometries are available including solvent traps or humidity control chambers especially suitable for testing water swollen gels. The basic measuring mode is shear with the control of the force in normal direction and precise gap measuring and setting Figure 18. The samples have then disk-like or cylindrical geometry. The majority of contemporary rheometers is equipped with the possibility to monitor the steady strain or stress responses in time, i.e. to perform the relaxation measurement leading to equilibrium modulus value and they are capable of shifting between strain controlled and stress controlled modes. To find the equilibrium modulus, the sample is in contact with the upper measuring plate. By turning only the upper plate around its central axes of a small angle, a small deformation is introduced on the sample. The rheometer reads the sample force response - the value of stress exerted by the sample when the plate is kept steadily in its new position. Under the load, the chains in the hydrogel will rearrange to spend minimum energy for holding in the new position. This process is called strain relaxation. The value of sample stress response that will not change in time is the value needed for finding the equilibrium modulus. A similar

experiment can be done in a creep mode. The plate is pushed to turn around the central axis with a constant force set before. The sample response is a certain deformation. When the final deformation is reached, the so called creep compliance or creep modulus can be calculated.

However, the basic mode in rheometry is dynamic, from which the value of moduli is not equilibrium but frequency dependent. Sometimes the frequency influences the modulus only negligibly so when the value is very close to the equilibrium modulus, the result from experiment is the “quaziequilibrium modulus”. When viscoelastic materials (as most of the polymers are) get loaded in a dynamic manner, typically with sinusoidal oscillations at set angular frequency ω , a very complete information about their structure can be figured out from the mechanical responses. The whole scientific field of mechanical spectroscopy of polymers has developed.

The sample response is divided into a real (in-phase, storage) contribution, and into an imaginary (out-of-phase, loss) contribution. The real part gives so called storage modulus, G' , which is controlled by elastic properties of material; and out-of-phase (loss) part, giving loss modulus G'' , which is a measure of viscous properties of material. For instance by application of the oscillatory strain $\gamma(t) = \gamma_0 \sin(\omega t)$ it is possible simultaneously and independently obtain the both moduli:

$$\sigma(t) = \gamma_0 (G'(\omega) \sin(\omega t) + G''(\omega) \cos(\omega t)) \quad (15)$$

The G' and G'' moduli are related according to equation:

$$G^* = G' + iG'' \quad (16)$$

In the oscillatory rheometer, a viscoelastic measurement of swollen hydrogels and polymers is usually performed in the parallel plate-plate or sometimes cone-plate geometries. Dependences of the oscillatory strain $\gamma(\omega)$ or oscillatory stress $\sigma(\omega)$ are typically plotted together with the phase angle $\delta(\omega)$ that characterizes the shift between the real and imaginary parts:

$$\begin{aligned} G' &= G^* \cos(\delta); \quad G'' = G^* \sin(\delta); \\ \tan \delta &= \frac{G''}{G'} \end{aligned} \quad (17)$$

For the rubbery materials with a fully elastic response, the phase angle δ will be 0° and for the purely viscous liquid (with no elasticity) the phase angle will be 90° .

The dynamic oscillatory mechanical spectroscopy is a powerful tool for characterizing the time changes in materials; very importantly for hydrogels: their structure build-up during crosslinking polymerization. A major contribution to this topic was the work of Chambon and Winter [47] published in 1985. In this paper, they first discovered that in the vicinity of the gel point (called rather a critical point), the time dependences of $G'(\omega)$ and $G''(\omega)$ plotted logarithmically were parallel. They presented convincing experimental results obtained with model polybutadiene polymer networks. The gel time thus can be well characterized using the multiple frequency rheometry.

An excellent introduction to viscoelasticity of polymers can be found in the comprehensive classical textbook by Ferry [48]. The details on rheometry, the painstaking description of experimental equipment technique with the theoretical background is available in the book by Macosko [49]. The issue of rheological characterization of polymer networks has been recently very clearly worked out with many important references in the textbook by Pascault et al. [50].

One practical drawback of the rheological characterization of a material is the relatively high volume of material needed for testing. Importantly, the rheology will not give quantitative results with porous hydrogels; it only describes the sample as a whole without taking into account its internal microstructure.

A curious and a promising method for hydrogel characterization is the so called microrheology. Microrheological methods have appeared in the year 1948, when the Einstein's theory of Brownian motion was proved experimentally by Jean Baptiste Perrin and when the mean-square-displacement (MSD) of microsized beads in viscous liquid was observed and measured using microscope for the first time. The historical background of the early experimental work as well as the recent development of the theory and techniques are comprehensively described in the review [51]. The microrheological methods experience their comeback in the present when much more sophisticated in-situ observation technique is available. The principal of microrheological measurement is simple; nowadays not one particle but rather an assembly of small particle-probes embedded in the tested material is being traced and the mean time displacement of particles is calculated from the time dependent image analysis and plotted as a time function. Usually as probes submicron sized latex particles that can undergo the Brownian motion (i.e. submicron size) are used. In a viscous liquid, the particles would travel apart each other in time without limitations, so the mean-square-displacement will steadily increase. With the growth of liquid viscosity (e.g. with gelation), the probes would become more and more fixed by the structure in a certain volume and finally, as the system goes through its gel point, the characteristic displacements of the probes will become constant. In the active microrheology, the particle movement in the sample is excited by the external force, such as the magnetic field. Knowing the external force extent and the displacement from measurement, the moduli of the sample can be calculated. The microrheology brings advantage mainly for biological materials science when often only small amounts of samples are available and can serve mainly as a semi-quantitative characterization of the gelation process. There is a well developed web site about the microrheology authored by the scientists of the Department of Physics of the Harvard University [52]. Theoretical fundamentals of microrheological methods are further reported in [53],[54].

The absolute values of moduli of the synthetic hydrogels in their fully reacted swollen state are very important factors informing about the possibility of *in vivo* gel use. Also, when cells are supposed to attach and to spread onto the artificial gel support; the mechanical properties of such support seem to be the key factor. It has been evidenced that the living cells feel and prefer the stiffness of the environment in which they proliferate [55]. The literature brings numerous examples of experimentally determined mechanical moduli of various tissues; typically the gel stiffness is described with compressive or shear moduli. Helmlinger et al. [56] studied brain tumor proliferation and proved that multicellular tumor spheroids can overcome the mechanical stress up to 6 kPa in an agarose gel before they become inhibited at stresses between 6 and 16 kPa. Yu and Schoichet [57] studied hydrogel scaffolds (prepared

from a porous synthetic hydrogel) for the growth of neurites from primary neurons in order to regenerate soft nervous tissue. They state values of compression modulus of such tissue to be around 200 kPa.

A homogeneous slightly crosslinked hydrogel based on the poly(2-hydroxyethyl methacrylate) prepared with 30 wt.-% of water in the reaction mixture as diluent had the relaxation (equilibrium modulus) measured in shear around 55 kPa. When the same hydrogel is made highly porous by increasing the dilution its total modulus decreases down to 2.5 kPa.

This part of the chapter could only serve as a brief introduction into the issue of hydrogel mechanical behavior and its characterization. To gain further knowledge, the reader might appreciate the very good review by Anseth [58] with its some fifty collected references.

4. CONTACT LENSES

Contact lens is a small optical device placed directly on the cornea. The technology of its production must allow tuning of optical properties, it must be possible to adjust shape precisely and ensure appropriate physiological function of lens in contact with the eye. The cornea is a transparent tissue with no veins that receives the nourishment mostly from the air oxygen. The artificial contact lens, however, always has some effect on the regular metabolism of the cornea. It is necessary to minimize the hypoxic and mechanical stress that may occur upon contact lens use.

The first idea to alter corneal power by optical system placed directly on the cornea was described by Leonardo da Vinci in the beginning of sixteen century [59]. His solution consisted in immersing the eye in a bowl of water. Next generation of similar constructs has been described by René Descartes in 1636 [60]. Twinkling of eye represented however an unsolved problem. In 1801 Thomas Young [61] constructed fluid-filled tube equipped with a glass lens, which was placed to the eye orbital rim. Sir John Herschel in 1845 proposed glass contact lens analogous to modern lenses, but the space between cornea and the lens was sandwiched with animal jelly [62]. More than forty years after this, the first real contact lenses of glass were made. The progress made of in the area was associated with the names of Adolf Eugene Fick (Switzerland), Eugene Kalt (France) and August Müller (Germany) in the late 1880's. The half walnut shaped lenses were made by glass blowing, they were scleral and afocal and they rather served as a partition between cornea and eye lid to prevent spreading of a disease mechanically. Their preparation, testing and application were described in detail by Adolf Eugene Fick in 1888 [63]. In the same year, a successful use of such lenses on human patients suffering from keratectomy was reported in France by Eugene Kalt [64]. First sanded glass contact lenses had defined optics and could correct the eye refractive error. Their inventor, August Müller used them to correct his myopia and he published his experience in 1889 [65].

Another significant progress in the contact lens development was made in 1936 when a new plastic, polymethyl methacrylate (PMMA), was brought to the market by Rohm and Haas company. In this year, William Feinbloom [66] reported on a scleral lens made from plastic and glass parts. Soon after, a first fully plastic lens was manufactured using lathe-cutting and polishing techniques. This was the beginning of the new whole area of industry.

The transparent synthetic polymers became the suitable class of materials for contact lens production.

During the World War II the PMMA was shown to be biologically inert and it was also less fragile compare to glass. These reasons together with easy processability caused that PMMA became a key material for contact lens production for several decades. Lathe-cutting allowed making lenses of any shape and optical power.

In 1950 Kevin Tuohy patented the hard corneal PMMA lens [67] used in its somewhat advanced version to treat certain conditions even today. They are known under the term rigid gas-permeable lens (RGP).

Over all the advantages, one drawback of PMMA plastic was that the material was not permeable for water neither for gases. That means that no oxygen or any water-soluble compounds could not penetrate through however the cornea needs to be doped by the air-oxygen permanently. If for longer time, the cornea suffers from hypoxic stress, the malfunctioning and serious damage appear. In the course of solving such problem of the material, the siloxane structures or perfluorated alkylmethacrylates within the PMMA chains were investigated [68].

Silicone elastomers are known for their high gas permeability including oxygen. Also, these soft materials have some mechanical parameters comparable to natural tissues. This was an important feature as it became known that the mechanical stress can be as serious as the hypoxic stress. On the other hand, the soft material could not be shaped by the lathe cutting; the mold pressing into the precise individual molds had to be used instead. Another drawback of silicone elastomers is their hydrophobicity. Water-soluble species will not penetrate easily through a silicone layer while the adhesion to the eye surface is very strong. Some patients even experienced severe damage of their cornea when removing silicone lenses from their eye. The highest number of patents of silicone contact lenses was filed around 1965 and the development went through its peak period in late seventies. But after all, for the disadvantages mentioned above, the silicone lens did not make its turn. Yet, it is still used but only sparingly; in cases that demand a special treatment.

The rigid gas-permeable lenses (RGP lenses, PMMA-based) are still favored and commonly used in many developed countries. The development of gas-permeable plastics still continues and the values of permeability for oxygen of the current RGP lenses are quite high while the surface wettability is adopted precisely to application. They are often manufactured to meet individual needs, i.e. tailored to each patient. They are still indispensable in the correction of high astigmatism and for clients with keratitis. Their development is historically associated with a number of patents by Norman Gaylord [69].

A remarkable achievement in the lens technology was the discovery of soft synthetic hydrophilic polymers, synthetic hydrogels, that were used for first lenses in their lightly crosslinked state [70], [71]. The polymeric synthetic hydrogels have the capability to absorb water up to certain equilibrium volume that is constant for given system and temperature (equilibrium swelling). Therefore, the hydrogel contact lens contains always some water that functions as a transport media for low molecular weight nutrient species and metabolites (oxygen, ions, etc.). Even though the number of users of the second and third generations of the silicone-hydrogel contact lenses is increasing remarkably and their market is undergoing steady growth, the methacrylate hydrogel lenses are nowadays the most common type of contact lenses. This is perhaps for the flexibility of their application regimes.

The polymer material for hydrogel contact lenses were developed in fifties by Otto Wichterle and Drahoslav Lim [70]. Professor Wichterle patented a basic material, poly(2-hydroxyethyl methacrylate), polyHEMA for production of hydrogel contact lenses together with a unique method of their manufacture; the spin-casting [71]. In 1963 Wichterle added a patent for lathe-cutting of soft contact lenses from a hard block of dried gel; xerogel. Although the first applicable contact lenses were spin-casted in 1961, the production was started in the USA in 1972 by the Bausch & Lomb Company.

Hydrogel materials integrate several important properties: they are soft, wettable, they are compatible with living tissue, they are partially permeable for gases, and they allow flow of water through their structure as well as diffusion of low molecular weight solutes. They also have some drawbacks. The original first hydrogel material, crosslinked polyHEMA, can swell in water at the laboratory temperature only up to about 40 wt.-% of water content in the gel-water system. Its permeability for oxygen is about 8-12 barrer (whereas the RGP material permeability can be 8-120 barrer and that of the silicone elastomers up to 200 barrer) [72].

Since their discovery, the hydrogels were further investigated with the goal of the equilibrium water content and permeability for oxygen increase. Thus, the copolymers of HEMA with other hydroxyalkyl methacrylates, methacrylic acid or its salts were prepared, cf. Figure 19. These hydrogels swelled in water up to 55-60 wt.-% of equilibrium water content while their *Dk* reached values in the range 20-25 barrer; nevertheless they were prone to attachment of protein deposits. The protein adhesion is promoted by the lower water content in the material surfaces and is stronger when there is a charge in the macromolecular chain of the substrate (cf., e.g. methacrylic acid and their salts). The higher water content reached materials based on the copolymers of N-vinylpyrrolidone with different alkyl methacrylates, or hydroxyalkyl methacrylates. These polymers can attach lipophilic deposits that are however more easily removable. The group of hydrophilic monomers for contact lenses includes many other molecules, e.g. glycerol methacrylate, glycidyl methacrylate, (meth)acrylamide, poly(vinyl alcohol).

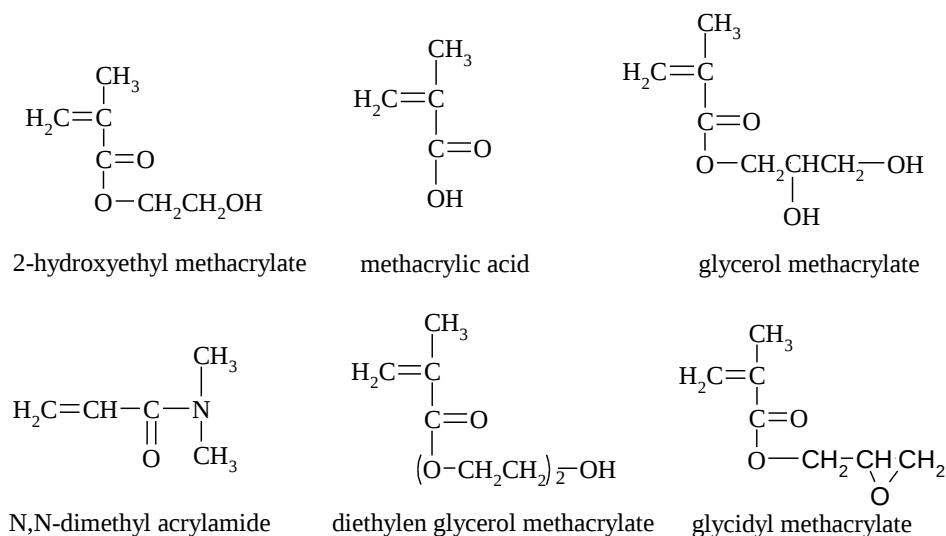


Figure 19. The chemical structures of methacrylate monomers.

The development of materials for contact lenses spanned over three decades. Within that time not only the chemistry but also the medical design of the lens body and the wear regime would be subjected to changes. In 1998, the first lenses with planned replacement were introduced to the market, Acuvue (J & J) and six years later, 1994, the first daily lenses were brought up (Bausch & Lomb) [73].

Since the beginning of seventies, the contact lens material design was driven by the idea to combine the advantageous properties of the silicone elastomers with the hydrophilicity and thus have a transparent, highly permeable, and swellable material with the capability to allow diffusion of small species. Various sandwiched materials were constructed or surface modifications were tested, the most common approach to that was hydrophilization of the silicones. Most of the attempts turned unsuccessful. The break through was seen at the end of the nineties. The research teams around the world would focus on making a co-continuous structure, silicone hydrogels [74].

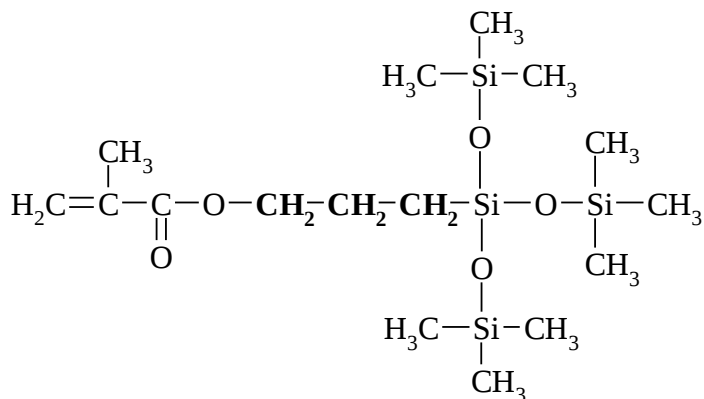
The high oxygen permeability is achieved with the monomer commonly referred to as "TRIS" (Figure 20A), which is known from RGP materials. The methylene groups in the structure of TRIS (in bold) represent the sites for hydrophilic modification. Material balafilcon A (PureVision CL) is based on polymer of vinyl carbamate derivative of TRIS (Figure 20B). Lotrafilcon A (Focus Night & Day) is copolymer of TRIS monomer with N,N dimethyl acrylamide and macromonomer B (Figure 20C) [74].

Second type of novel silicone-based materials contains oligomers bearing short blocks of hydrophilic chains, oligosiloxanes, and/or perfluorated chains in their backbones. Not only the copolymers themselves were subjects of the patents but also the methods of their processing, e.g. the methods necessary surface modifications to ensure uniform wettability of lens surface, i.e. methods leading to elimination of hydrophobic domains.

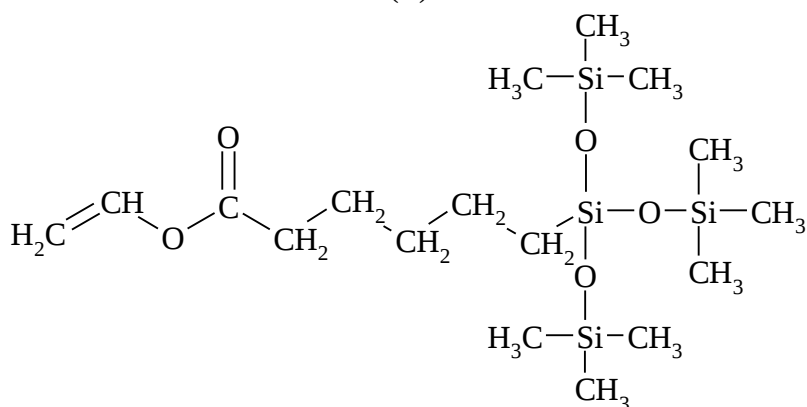
In 1998, the first brands of silicone hydrogel contact lenses entered the world market: Focus Night & Day by CibaVision Company and PureVision by Bausch & Lomb. Application of these materials in the end-use products has set another milestone after the invention of the synthetic hydrogel contact lens by Wichterle. The advanced silicone hydrogels achieve swelling values comparable to standard hydrogels, their permeability for oxygen is lower but their surface hydrophilicity is enhanced (wettability), their Young modulus is lower - these are silicone hydrogels of the 2nd and 3rd generation.

The current trends in the field of standard and silicone hydrogels focus on inclusion of a highly hydrophilic linear monomer bound into their molecular structure by physical interactions instead of covalent bonds. For example, such interactions can have even topological cause - they can be molecular entanglements that prevent the molecular chains from diffusing out of the structure (e.g. by washing) because of their high molecular weight and sterical hindrances. The hydrophilic chains bind firmly water molecules and thus prevent the whole system from drying out while worn.

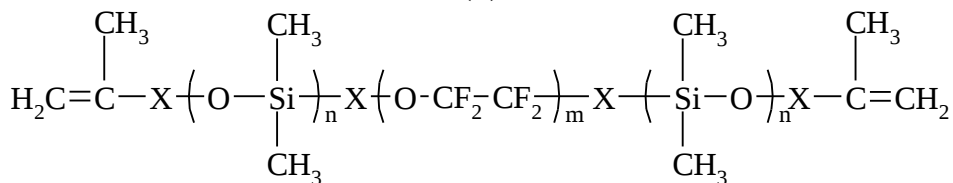
The contact lenses are modern, sophisticated, and when properly used, a safe vision correction aids. Although a number of materials were described in this chapter, the standard synthetic hydrogels still represent the most typical material for soft contact lenses while the contact lens has been a long-term successful most spread application of hydrogels in biomedical area.



(A)



(B)



(n = 5 -100, with advantage 14 - 28; m = 10 - 30)

(C)

Figure 20. The examples of chemical structures of monomers for silicone hydrogels.

5. INTRAOCULAR LENSES

Intraocular lenses (IOL) are implanted into the eye in order to correct vision. Their application represents one way of refraction correction or they are used in the cases of aphakic eye resulting from cataract surgery. Cataract operation consists of several phases. A cloudy natural lens is removed and subsequently replaced by a synthetic lens. The IOL

parameters have to be precisely adjusted according to the previous measurements of patient's eye [75],[76].

Intraocular lenses can be implanted into the various parts of the eye, e.g. into the interior chamber, the posterior chamber, or the stroma. The implantation locus determines the lens shape parameters. The two main designs of IOLs in current use are the interior chamber IOL and the posterior chamber IOL [76]. Every IOL consists of two parts - an optical part and haptics anchoring the lens. Anterior chamber IOLs lie in front of the iris and have a flexible or semiflexible angle-supported haptics. Implantation of these IOLs is possible for both intracapsular and extracapsular cataract extraction. They are useful as a standby if the posterior capsule was accidentally ruptured (i.e. posterior chamber implantation is not possible). Posterior chamber IOLs lie behind the iris and have flexible haptics which are inserted either into the capsular bag or into the ciliary sulcus [77].

Material for IOLs should fulfill various requirements such as the proper mechanical properties, the refractive index, the glass transition temperature and the biocompatibility. Two groups of materials are used for manufacturing of IOLs – silicones and polymers of acrylic acids. The group of acrylate polymers can be divided into the rigid (hard) materials, such as polymethyl methacrylate (PMMA), and the soft/foldable materials, including hydrophobic and hydrophilic acrylics. The main advantage of soft materials is the possibility of their deformation by folding and subsequent implantation through a very small incision. During the implantation, the lens is placed into the capsule, then relaxes and reaches the previous shape. With haptics, it is fixed in the optical axis of the eye. During the IOL development, the production volume of soft materials dramatically increased compare to hard acrylics due to the possibility to implant soft lenses through significantly smaller incisions with the advantage of lower number of the post-operation complications. However, small or even tunnel incision is more difficult to manage and is more dependent on the instrumentation; that means that this way is more expensive and therefore more common in the rich and industrialized countries. So, the population of the “third world” countries maintains the number of implanted conventional PMMA IOLs still at a high level. Besides the foldable hydrophobic acrylates, e.g. Acrysof® (Alcon Laboratories, Fort Worth, Texas, USA) or Sensor® (Advanced Medical Optics, Santa Ana, California, USA), with the content of water lower than 1 wt.-%, hydrophilic copolymers containing 18-38 wt.-% of water are used [78],[79], e.g. MemoryLens® (Ciba Vision, Duluth, Georgia, USA), Centerflex® (Rayner Intraocular Lenses, Brighton-Hove, East Sussex, UK) and Hydroview® (Bausch & Lomb, Rochester, New York, USA). Hydrogel IOLs are prepared for example from slightly crosslinked network formed by the terpolymer: 2-hydroxyethyl methacrylate-co-ethylene dimethacrylate-co-methacrylic acid transferred into sodium form [80].

In the case of hydrogel IOLs, the deformation prior to implantation may be fixed and intensified by partial drying and/or cooling. After the implantation, partially dried hydrogel lens increases volume due to the swelling in eye liquids and/or increasing temperature (35-36 °C). Another advantage of acrylic materials is higher refractive index compared to silicones which enables thinner construction of the lenses yet maintaining good refractive properties [79]. The surface of hydrogel IOLs can be modified in order to reach better biocompatibility and to avoid inflammatory reactions after the implantation [81],[82]. The retina can be protected by chromophores absorbing UV light, such as benzotriazole and benzophenone, added to the IOL material [79]. Hydrophilic acrylic material used for

preparation of IOLs can also serve as a drug delivery system, e.g. as carriers of antibiotics preventing post-operative complications [83].

The response of the surrounding tissue to the IOL implantation and consequent complications were extensively studied [84],[85]. The influence of hydrophilic and hydrophobic materials, as well as IOL shape, on the creation of secondary cataract as the most common post-operative complication was observed [81],[84]. These studies emphasize the importance of shape, while other source [86] referred that hydrogel IOLs show five times lower occurrence of the secondary cataract in comparison with PMMA IOLs. Also, the reaction of the IOL hydrogel material on the surrounding tissue was investigated [87] and processes such as opacification [88] and calcification [89] were interpreted. Presence of carboxyl groups (especially on the material surface) plays a positive role in decreasing of calcification probability [90].

Development of IOLs has proceeded from relatively simple PMMA implants via flexible materials implantable through a minimum incision to the lenses with more sophisticated optics, e.g. toric IOL correcting astigmatic vision (AcrySof® Toric, Alcon Laboratories, Fort Worth, Texas, USA).

The next important achievements in IOL development are accommodating IOLs, which could provide excellent vision at all distances (far, intermediate, and near) without any side optical effects such as unwanted retinal images, halos, glare, and loss of contrast sensitivity. Moreover, they have the potential to reduce the patient dependence on glasses after cataract surgery [91].

As other advancement, it is necessary to mention the development of light-adjustable IOLs, which affords the opportunity to correct post-operative refractive errors [92]. After IOL implantation, the use of light exposure may initiate polymerization of remaining unpolymerized subunits in the lens material or additional material crosslinking in a precisely defined area resulting in a change in the overall optical lens power.

The future in IOL, as in other areas of synthetic implant applications, is associated with self-assembling systems, it means with the liquid injection systems, which can form pre-defined structure in the desired location. However, cataract operation nowadays represents ordinary matter, which concerns a wide range of populations, particularly in the context of increasing number of refractive surgical interventions. The manner of its implementation, including the selection of implantable lens material, is significantly influenced by the economic possibilities of the health system, respectively patient.

6. FUNCTIONAL IMPLANTS

Hydrogels for Urinary Incontinence Treatment

Urinary incontinence causes serious medical and social problems, having a deep influence on a patient's psychological state and causing a series of direct social impacts. The management of incontinence is very expensive; the cost in USA in 2000 exceeded 30 billion USD [93]. All current surgical methods of incontinence treatment are based on the increase of urethral resistance at the site of injured or missing sphincter in males and in females with intrinsic stress incontinence. Drugs can not effectively modify the pressure of an injured

sphincter in cases of serious muscle damage. The approaches to the surgical procedures in such patients are based on the increase of urethral resistance in the sub-vesicle area by the application of teflon, collagen, or silicone particles [94]-[96], the sling [97], the artificial sphincter [98] or the urinary catheterization [99]. Such artificial obstructions are aimed to prevent spontaneous leak of urine, whenever the abdominal pressure increases; on the other hand, it has to enable free passing of urine without residuum.

The use of hydrogel implants in incontinence treatment brings very promising results [100], [101]. The method consists in subcutaneous implantation of a cylindrical dry hydrogels (Figure 21a) to the damaged area of sphincteric part in bulbomembranous urethra. The number of implants is chosen depending on the extent of incontinence and the swelling degree of implant is adjusted. Dry implants get swollen by extracellular liquid and thus create the obstruction, which prevents accidental escape of urine (Figure 21b). The resistance to the urine flow corresponds to the pressure of 20-25 cm of water column in the urinary bladder. Thus, the generated subvesical obstruction permits a spontaneous bladder emptying without residua. The implant is prepared from a strongly hydrophilic hydrogels, such as the copolymers of 2-hydroxyethyl methacrylate (HEMA) with sodium methacrylate [100], or acrylonitrile with acrylamide [101]. The clinical studies [102] showed the significant improvement of incontinence for 17 women (age ranging between 35 and 84 years), while about a half of them experienced a very significant improvement of incontinence.

Spherical hydrogel microparticles represent a type of modified hydrogel implants [103]. Their size ranges from 30 to 300 μm and they are applied in the dry state by injection into a designated location. After swelling, they create the demanded obstruction. However, the clinical effects tend to disappear after several months (in contrast to cylindrical implants mentioned above), either due to migration of the particles away from the injection site (caused by weak adherence with the surrounding soft tissues) or due to fibrosis (caused by excessive encapsulation of the particles by fibrous tissue). Little is known about the fate of injected microparticles, due to the fact that they are extremely difficult to trace in a noninvasive manner.

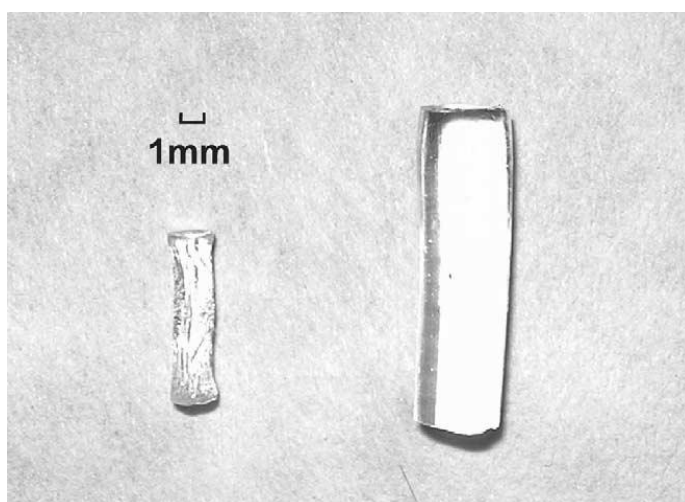


Figure 21. Hydrogel implants before (a) and after (b) 24 h in saline.

7. BLOOD VESSEL EMBOLIZATION

Embolization of blood vessels has emerged as a highly effective technique in a wide variety of diseases. The method is based on keeping the embolic region away from blood circulation. But the efficiency of the method, and thus the degree of devascularization, was found to be limited by the development of a new vascular network around the pathologic area, which has the tendency to nourish the isolated region [104]. Such revascularization is associated with an inflammatory reaction of giant cells (macrophages), as it was observed by histological investigation [105]. The most promising results in clinical practice for the obliteration of blood vessels such as uterine arteries in the treatment of uterine fibroids have been obtained using calibrated, soft, compressible, and relatively hydrophilic microspheres of controlled shape and dimensions [106]. Since 1995 up to 2007, more than 50,000 cases of uterine fibroid embolizations, which are the most common tumors in the female genital tract, were performed worldwide [107]-[109]. Embolization is also used for treatment of inoperable tumors [110], arterio-venous malformations [111], craniofacial vascular malformation [112] and haemoptysis (excessive bleeding) [113], [114]. It is appropriate to modify embolization material by any roentgen-contrast compound in order to check the embol position roentgenologically.

Three ways of embolization are applied:

1. Use of a homogeneous, for example ethanolic solution of polymers (e.g. polyHEMA) insoluble in water, respectively in the blood [115]. When such solution is applied into the vessel, polymer precipitates and obstructs the vessel. This system allows dissolving a roentgen-contrast substance in ethanolic solution of non-crosslinked polyHEMA which is insoluble in the blood and remains in embolus of polyHEMA. In similar manner calcium alginate can be used [116].
2. Use of the hydrogel microparticles dispersed in saline. The mechanical obstruction is formed upon the injection of microparticles into the vessel. The advantage of regular microspheres over irregularly shaped particles is that the uniform geometrical shape allows precise location of the embolic material. Further improvement of the microspheres can be achieved by biofunctionalization of the surface. By immobilization of a coagulation factor, such as thrombin, on the surface, the blood coagulation can be triggered. The advantage of this way for embolization therapies is that the spheres get anchored. Suitable hydrogel for microparticles proved to be the terpolymer of methyl methacrylate, methacrylic acid and 2-[4-iodobenzoyloxy]-ethyl methacrylate [117] crosslinked by the tetraethylene glycol dimethacrylate. Comonomer containing iodine serves as a roentgen-contrast agent, methacrylic acid allows the bounding of coagulation agent (thrombin) and methyl methacrylate improves the mechanical properties of hydrogel. Microparticles are prepared by dropwise addition of the mixture of monomers and initiator (benzoyl peroxide) to the aqueous solution of polyethylene glycol, polyvinyl alcohol and polyvinylpyridine under stirring at 85 °C. After polymerization, the microparticles are washed with water and lyophilized. Thrombin is covalently bound by its amino group to the carboxylic group of a hydrogel. Due to the good adhesion of thrombin to the walls of a blood vessel of laboratory mice, compact embol is created and its position and

shape in the vessel is easily radiologically observed. Crosslinked microparticles of poly(acrylamide) known under the commercial name of Trisacryl® can be used in a similar manner [118].

3. Use of the metal wires, cylinders or spheres coated by a hydrogel [119]. This method is a modification of the previous method and its advantage is in the easier shape adjustability of the embolization implant.

8. WOUND DRESSINGS

Extensive burn injuries and other large skin defects of traumatic and/or metabolic nature are a serious medical, social and economic problem and the management of wound healing process still represents a challenge for current research in the medical area. The variety of wound types (burns, diabetic leg ulcers, pressure ulcers, surgical wounds, and etc.) has resulted in a wide range of possibilities of using different types of wound dressings to the successful management of the therapeutic process. Wound dressings have undergone a considerable evolution from crude application of plant herbs, over traditional dressings (cotton wool, natural and synthetic bandages and gauzes), that simply cover and conceal the wound, to modern materials ensuring the moist healing process, and more recently, to advanced tissue engineered scaffolds, which can replace the damaged skin.

Perhaps one of the most important advances to change the nature of wound dressing materials has been the confirmation of the importance of a moist environment around the wound to facilitate the healing outlined by Winter in 1962 [120]. Since then, the concept of the moist wound healing has been largely examined, which has led to the development of hundreds of modern wound dressings that support a moist wound environment, the results of which have been reviewed elsewhere [121]-[126]. Examples of commercial products available on the market today include hydrocolloids Granuflex™ (Conva Tec, UK), Comfeel™ (Coloplast, USA), or Tegasorb™ (3M Healthcare, UK), alginates Sorbsan™ and Tegagen™ (3M Healthcare, UK), and hydrogels NuGel™ (Johnson & Johnson), Purilon Gel™ (Coloplast, USA), or IntraSiteGel™ (Smith & Nephew).

Hydrogels play in the area of wound dressing an important role as they meet the most requirements for an “ideal” dressing due to their ability to contain significant amount of water (up to 90 wt.%), that guarantees the maintaining of moist environment together with the possibility to absorb excess of exudates. Moreover, they are nonirritant and well tolerated with living tissue, permeable to metabolites, cause no trauma on removal due to their elastic character, and they cool the surface of the wound, which may lead to a marked reduction in pain and therefore high patient acceptability.

A detailed discussion about application of hydrogels in wound healing is, however, beyond the scope of this review. Therefore, in the following paragraphs we will focus on the methacrylate hydrogels applicable in wound healing being developed in our research group, i.e. hydrogel dressing materials containing radical scavengers and hydrogel supports suitable for keratinocyte cultivation for skin damage repair.

8.1. Hydrogels Containing Radical Scavengers

It is known that during the healing process a large number of different free radicals is formed in wound with the task of local disinfection. However, the free radicals besides protection from foreign micro-organisms also destroy the own cells and thus slow down the process of wound healing.

This negative aspect can be solved by incorporation of radical traps into the hydrogel structure. Such hydrogel dressing then shows the synergic effect of moist healing and deactivation of the free radicals. We have patented the preparation of slightly crosslinked hydrophilic polymer support based on methacrylic acid derivatives, in which biologically active compounds with radical catcher properties (derivatives of the vitamins A and E) were dispersed or dissolved in polymer matrices in amount up to 50 wt.-% [127]. It has been demonstrated that the wounds treated with this type of dressings healed without any secondary infection and successful epithelialisation was observed.

Another developed material belonging to group of products for the wound treatment is methacrylate gel containing in its structure covalently bonded sterically hindered amines, known for their ability to neutralize free radicals and thus facilitating the healing process [128]. This product is nowadays available on the market under the name HemaGel produced by Wake Pharma company.

8.2. Hydrogels as Cultivation Supports

The tissue-engineered skin represents a recent significant advancement in the wound healing. A number of products is nowadays commercially available; particularly IntegraTM, AllodermTM, or ApligrafTM [121], [129] and many others are currently being developed.

The method of keratinocyte cultivation and subsequent transplantation was developed in the USA in 1979 [130]. It is predominantly used to re-epithelialisation of burns or wounds. Using this methodology, it is possible to prepare several thousand square centimeters of epidermal sheets in 3-4 weeks. Cultivation of autologous cells proceeds under the tissue cultures in the presence of 3T3 mice fibroblasts (feeder cells), in whose the proliferation was stopped by irradiation. The confluent growth of keratinocytes is detached from the bottom of cultured Petri dishes by the enzyme dispase and transferred on vaseline gauze applied on the patient's wounds. The cell transfer is however technically demanding and it is often the cause of failure.

Therefore, the method allowing the direct cultivation of keratinocytes on a polymer support and its direct transfer to the wound was developed [131]. For the hydrogel support, the poly(2-hydroxyethyl methacrylate) (polyHEMA) was tested as a material with a long tradition in its biomedical use and a wide scale of applications. The process of keratinocyte cultivation is schematically shown in Figure 22. Firstly, polyHEMA supports were preincubated in bovine serum with a cocktail of proadhesive proteins (A) and then the lethally irradiated 3T3 mice fibroblasts were seeded to polyHEMA (B). After adhesion and covering the supports with feeder cell network, a suspension of keratinocytes was added (C). In seven to ten days keratinocytes formed a subconfluent to confluent growth and destroyed the feeder cells (D). The damaged feeder cells were removed by trypsinization and the support with grafted epidermal cells was directly applied in an upside-down position to the wound bed (E).

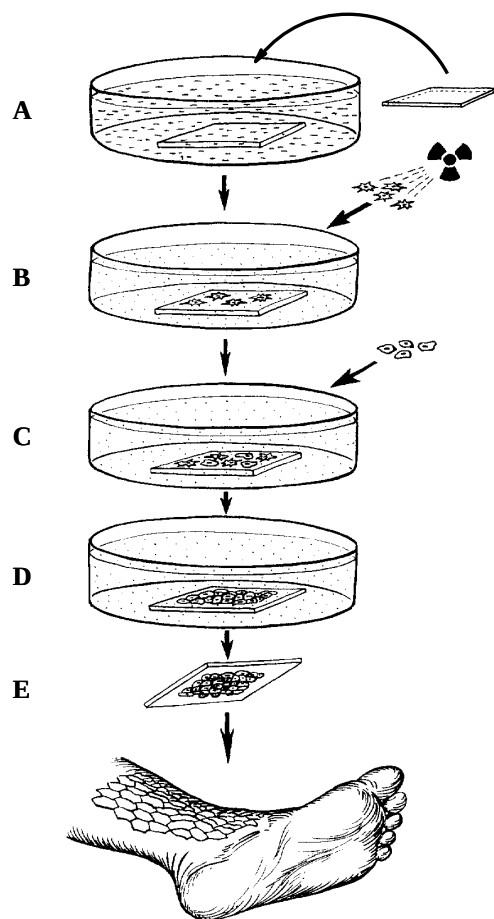


Figure 22. Scheme of keratinocyte cultivation on polyHEMA hydrogel supports and their application to the wound bed [131]. A – preincubation of polyHEMA in bovine serum, B – seeding of lethally irradiated 3T3 mice fibroblast, C – seeding of keratinocytes on the polymer support covered by network of feeder cells, D - keratinocyte proliferation, E – direct application of the support to the wound bed.

The results of clinical trials in a number of burned patients were highly encouraging [131], [132]: the cells, grafted upside down in a monolayer, migrated from the support and colonized the wound. This method, in comparison with classical procedure of the keratinocyte sheets on textile supports [130] is much easier; no enzymatic detachment of cells is necessary; the time necessary for the preparation of grafts is shorter, because the subconfluent growth of cells can be used; the hydrogel sheet on the surface of the wound bed protects the wound bed with transplanted cells and optimizes the microclimate.

Although the described upside-down technology brings desirable healing effect, the need of feeder cell used for initial attachment and growth of keratinocytes represents a certain complication from the point of view of clinical application. So, the exclusion of feeder cells would lead to the more effective process of keratinocyte cultivation, and moreover, the patient's immunologic burden would be reduced.

In the course of systematic study of biological properties of synthetic methacrylate hydrogels as potential supports for keratinocyte cultivation, we have observed that poly(2-ethoxyethyl methacrylate) (polyEOEMA) as a component of cultivation support stimulates the growth of human keratinocytes under *in vitro* conditions without feeder cells [133]. After 7 days, the surface of polyEOEMA was colonized with nearly confluent or confluent monolayer of cultured keratinocytes with extensive intercellular contacts. Moreover, keratinocytes cultured on these surfaces were able to migrate to the model wound bed *in vitro*, where they formed distinct colonies and had a normal differentiation potential confirmed by keratin immunohistochemistry.

This procedure is inexpensive and technically easy and therefore it seems to be suitable for keratinocyte culture in large scale and applicable for cell therapy of skin defects.

In recent years, advanced approach to eliminate the need of feeder cells for keratinocyte cultivation has received much attention, i.e. the preparation of cultivation supports modified by suitable bioactive pro-adhesive motifs, which would promote the adhesion, growth and proliferation of cells.

The preparation of bioactive polymer supports with covalently bound saccharides was patented [134]. Polymer is treated with a modification agent to activate the functional groups on the polymer surface (e.g. hydroxy, amino, carboxyl groups) and then it is allowed to react with appropriate derivative of saccharide (e.g. mannose, galactose, or lactose).

A desirable role of mannosides on the growth of human keratinocytes under *in vitro* conditions was recognized [135]. Mannosylated hydrogels were chemically synthesized and the successful cultivation of keratinocytes without feeder cells on such bioactive supports was demonstrated in *in vitro* experiments. These results offer a promising perspective; nevertheless the method is still too expensive for large-scale keratinocyte cultivation for the clinical practice.

It can be concluded that the modification of polymer supports by biologically active motifs is suitable for specific cell cultivation. So, the possibility of application of such polymer supports in the large-scale production will certainly be further investigated.

9. CONDUCTIVE HYDROGELS FOR BIOMEDICAL USE

Electrical conductivity of the hydrogels can be achieved by introducing of any ionizable monomers as salts of acids (acrylic, methacrylic, itaconic, sulfopropylmethacrylate, vinylsulfonic, styrenesulfonic, aminoacids), or monomers with quaternary aminogroup (N,N,N-trimethylammoniumstyrene chloride, methacryloyloxyethylammonium chloride, N-methyl-3-vinylpyridinium chloride, [2-(methacryloyloxy)ethyl]dimethyl-(3-sulfopropyl)-ammonium chloride) into a polymer chain. Conductivity is a function of the amount of ionizable monomer in the polymer chain and of the acidobasic properties of the system [135]-[139]. The use of homopolymers containing ionizable monomers is usually unsuitable for their very high swelling degree in water (water content at equilibrium swollen hydrogel is often above 95 wt.-%) followed by poor mechanical properties. Another possibility is the use of non-ionizable hydrogels, in which some conductive particles (salts, metals) are dispersed either touching each other or the contact between particles must be mediated through a conductive liquid electrolyte swollen into hydrogel [140].



Figure 23. Adhesive conductive hydrogel on ECG electrode.

In biomedical field, the conductive hydrogels are used mainly as biosensors [138]-[139],[141]-[143], ECG, EEG, defibrillation or high-frequency electrodes for electrosurgery, materials for tissue engineering [144] or for the enzymes detection [145]. In this chapter we will focus only on the application in electrodes.

Electrodes based on hydrogels are used in medicine predominantly for electrosurgery [146],[147], ECG and EEG [148] measurements and defibrillation [149]. In these applications, the use of conductive hydrogels with a good adhesion to the skin is usually required (cf. Figure 23) [150].

Such a hydrogel can be prepared by crosslinking polymerization, initiated by UV radiation or oxidative-reductive system, of moderately hydrophilic monomers such as the 2-hydroxyethyl methacrylate (HEMA), in combination with the ionogenic sodium methacrylate and in the presence of water and non-volatile hydrophilic solvents such as polyethylene glycols or glycerol. Conductivity is affected by the presence of sodium methacrylate dissociated in water. The adhesion power is given by the presence of hydrophilic solvent in sub-equilibrium concentration compare to free equilibrium swollen hydrogel; this guarantees that no components of hydrogel remain on the skin after removal of the electrode. Electrode is built from a metal foil connected to a diagnostic device (ECG, EEG), or in other instances to the source of electrical power, adhesive conductive hydrogel layer and protective foil that prevents hydrogel drying during the storage. The foil is removed immediately before use and the hydrogel layer is placed directly on the skin. Typical examples of such electrodes are products of Leonhard Lang company [150], named SKINTACT[®] (Figure 24-26).



Figure 24. SKINTACT Easibeat Multifunction Defibrillation Electrodes designed for the use of defibrillation, non-invasive pacing/synchronized cardio-version and monitoring.



Figure 25. SKINTACT ECG electrode.



Figure 26. SKINTACT Electrode for electrosurgery.

10. HYDROGELS IN TISSUE ENGINEERING

The area of tissue engineering (with cell therapy, drug delivery systems, and genetic engineering) is one of the most advancing biomedical disciplines especially because it offers treatment options where traditional medicine fails. The hydrogel materials play in this field a key role: as they enable the preparation of variety of scaffolds essential in tissue engineering. As this area is very wide, in this paragraph we will focus only on the applications of hydrogels in the central nervous system (CNS) and in particular in the spinal cord.

Brain or spinal cord injury causes severe tissue damage resulting in a permanent neurological deficit. Spontaneous regeneration is severely limited. The glial scar, mesenchymal scar and posttraumatic pseudocysts form an obstacle for regeneration. Tissue engineering continues to assume greater importance in neuroscience, with the ultimate goal of restoring the morphology and function of damaged nervous tissue. Tissue engineering techniques help to create a permissive environment to promote the regeneration of neurons and axons, oligodendrocytes, and blood vessels.

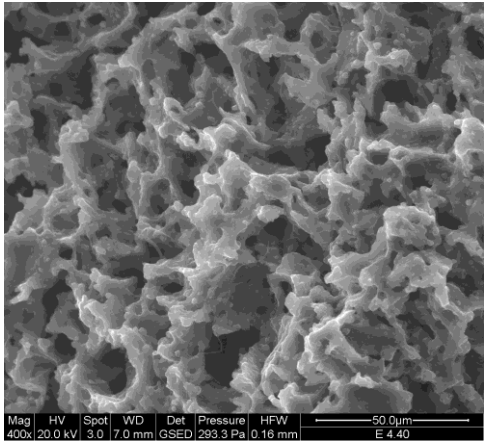


Figure 27. SEM micrograph of the typical morphology of macroporous hydrogel based on EOEMA/HPMA.

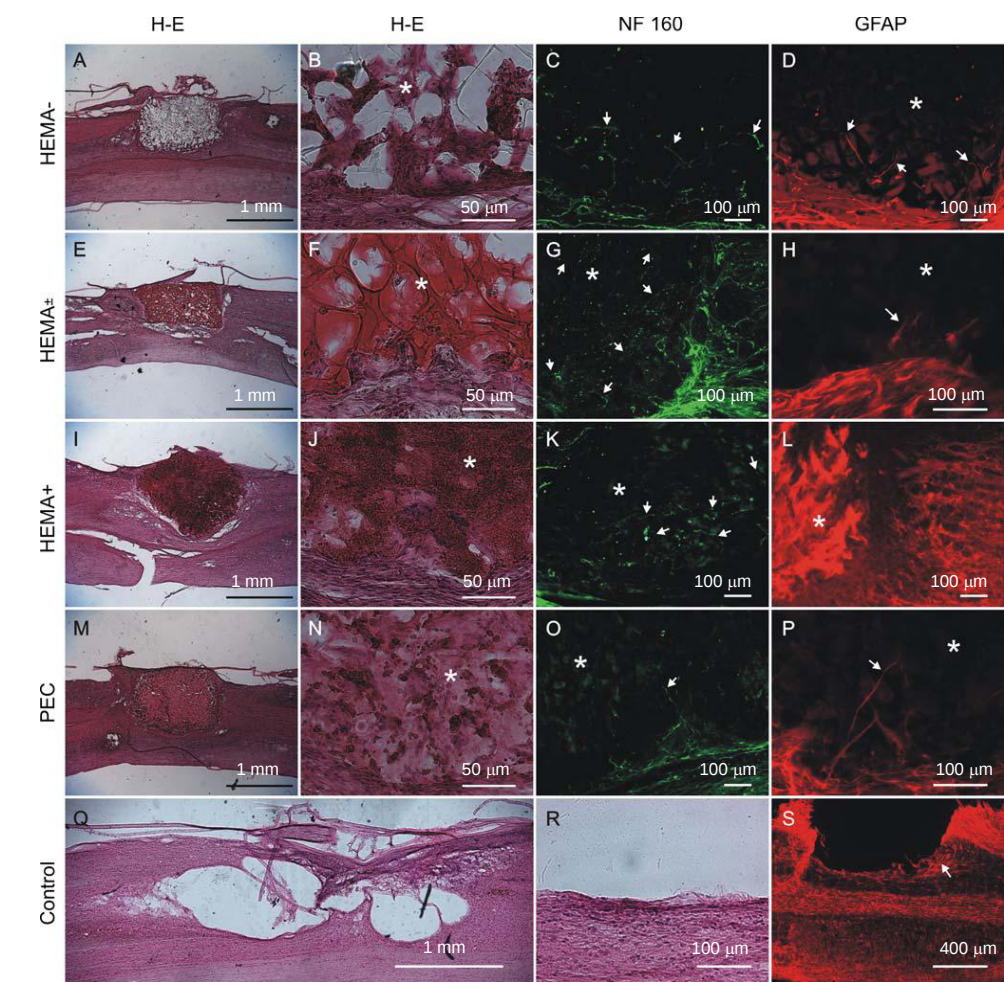


Figure 28. Tissue infiltration in the four HEMA-based hydrogels with different charges [161].

Various biomaterials, including hydrogels, have been implanted inside a CNS in order to bridge the lesion [151], [152]. Hydrogels have many advantages over some alternative scaffold materials, such as high oxygen and nutrient permeability and low interfacial tensions. The latter attribute minimizes barriers to cell migration into the scaffold from the surrounding soft tissue. The hydrogel scaffolds used in neural tissue engineering are commonly macroporous (typical morphology is shown on Figure 27) or become macroporous upon degradation, to allow room for neurite outgrowth. From this point of view, the pores must be communicating or prevalingly communicating as was described before [153]. Therein, other characteristics regarding morphology as total porosity, pore size, etc. are also referred. Further, the influence of both, positive and negative charge incorporated into polymer network on resulting structure of macroporous hydrogels was studied and reported in [154]. Preparation of macroporous hydrogels based on various methacrylate monomers (e.g. HEMA, HPMA, EOEMA) using pH sensitive and hydrolytically degradable *N,O*-dimethacryloylhydroxylamine as a crosslinker was studied and the possibility of adjusting the degradation time between 2 and 42 days was showed [155].

The mechanical properties of hydrogels are similar to those of soft tissue so that the load on cells in the area will be distributed normally and cells within the gel receive appropriate structural growth cues. The elasticity of hydrogels is also tunable by controlling the macromolecular structure and crosslink density. Another advantage of many hydrogels is that they easily conform to any defect shape, whilst they can be functionalized to include neurotrophins for the control of neuronal cell adhesion, proliferation and axonal extension. Cell replacement therapies in the CNS may also greatly benefit using hydrogels that provide artificial 3D stem cell niches for controlled proliferation and differentiation - an area of considerable activity in regenerative medicine. Diffusion parameters within implanted hydrogels attain values similar to those of developing neural tissue [156]. The physical and chemical properties of hydrogels can be modified to improve cell adhesion and tissue regeneration. Further, the synthetic hydrogels can be produced in large quantities, and combined with allogeneic or autologous transplants. Previous studies of ours and others have shown that hydrogels based on HEMA are promising biomaterials for CNS regeneration [151], [153]-[158]. Increasing knowledge of the pathophysiology of CNS, cell adhesion properties, molecular biology, and biomaterial science could lead to the development of implants that would successfully bridge a spinal cord lesion and lead to a complete recovery of locomotor, sensory, and autonomic functions. It is well known that the physical and chemical properties of a surface can influence cellular adhesion [159]. Previous studies have shown that neurons preferentially adhere to and form neural networks on positively charged surfaces such as polylysine-coated glass slides [160]. In our previous study, we found that HEMA-based hydrogels with positively-charged functional groups promote the adhesion of mesenchymal stem cells [157] that promote protection against tissue damage in experimental SCI and can increase the expression of growth and trophic factors in the ischemic rat brain.

The Figure 28 shows the tissue infiltration in the four HEMA-based hydrogels with different charge [161]. In the negatively charged hydrogels (HEMA-, copolymer HEMA with sodium methacrylate), there was only a small amount of connective tissue in the hydrogels visible on hematoxylin-eosin stained sections (A, B). The amount of connective tissue elements increased in hydrogels with both positive and negative charges (HEMA $\pm$, i.e. the terpolymer of HEMA with methacryloyloxyethyl-trimethylammonium chloride and with sodium methacrylate) (E, F) and was highest in the positively charged hydrogels (HEMA+,

copolymer of HEMA with methacryloyloxyethyltrimethylammonium chloride) (I, J). In the polyelectrolyte complex (PEC, complex of poly(sodium methacrylate) with poly(methacryloyloxyethyltrimethylammonium chloride)) implants, minimal connective tissue elements infiltrated the implant (M, N). The pseudocystic cavity dominated the lesion after hemisection without hydrogel implantation (Q), with a sharp border between the pseudocystic cavity and the residual tissue (R). Neurofilaments (arrows) were found in hydrogels with functional groups (C, G, K) in contrast to PEC implants (O). We found axons infiltrating the peripheral parts of the implants in all hydrogels with functional groups (C), while hydrogels with positive charges (G) had many axons also infiltrating the central parts of the implants. Few astrocytic processes were found especially in the HEMA- (D) and PEC implants (P) as compared with minimal astrocytic processes in the HEMA $\pm$ (H) and HEMA+ (L). The astrocytes formed a glial scar (white arrow) around the hemisection cavity (S). Hydrogels are marked with an asterisk.

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Chapter 4

BIOMATERIALS IN DENTISTRY AND MEDICINE

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ABSTRACT

The widespread use of biomaterials in medicine and dentistry is a relatively new phenomenon dating back to the 1950's yet, today, an estimated 20 million individuals have an implanted medical device.

Despite the huge impact that biomaterials have had on patients' quality of life, improvements in device performance and the development of alternatives to augment available therapies are continuously being sought. Clinical demand, advances in molecular and cell biology and the increased understanding of the role of the tissue-material interface on clinical performance has led to a metamorphosis of the biomaterials' field over the past 25 years. This has resulted in a change in the nature of biomedical devices from being biologically passive to actively integrated.

This chapter explores the development and application of biomaterials over the past 25 years, examining the current clinical demand, the scientific rationale, and the technical challenges to be overcome. As biomaterials are applied in reconstructive surgery and tissue regenerative therapies, these areas are explored with specific examples of recent developments and current research activity used to illustrate the changing perspectives.

Keywords: Biomaterials, Regenerative medicine, Tissue engineering,

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INTRODUCTION

Surgical intervention is not always required when tissue is damaged because of the human body's ability to activate the wound response following tissue trauma. The site and magnitude of the tissue injury, however, does dictate the extent to which the original tissue architecture and functionality is restored. For example, minor injuries to bone and epithelial skin do not require intervention as these tissues retain the ability to spontaneously regenerate in a near like-for-like manner, whereas injury to other tissues (*e.g.*, articular cartilage, the pancreas, the spinal cord, the dermis of the skin, brain tissue, neural retina, cardiac muscle, lung or the kidney glomerulus) results in the formation of scar tissue which replaces the lost tissue mass but does not restore tissue architecture or biological functionality [1].

When there is gross, acute or chronic tissue-dysfunction because of extensive traumatic injury or disease (*e.g.* spinal-cord injury or heart disease) surgical intervention to repair or replace the affected tissue is required. The options available to the surgeon include replacement (transplantation), reconstructive or, in a few cases, regenerative surgery [2] but are largely determined by the extent of tissue damage, the anatomical location and function of the tissue and the age and general health of the patient. Millions of patients have benefited from these approaches but many of these treatments fall short of their clinical requirements and may also be associated with the onset of secondary diseases. For example:

1. Replacement or transplant surgery relies on the excision of the dysfunctional tissue or organ and its replacement with viable tissue or organ. The transplanted tissue is generally an autograft (within one individual from one site to another) or homograft/allograft (between different individuals of the same species). Autogenous tissue transplants are used for bone grafts, full-thickness skin grafts, microvascular grafts and arterial-by-pass grafting and remain the 'gold standard' as they typically produce superior clinical results [3-7] *e.g.*, 60 % of bone grafts required in spinal fusion surgery are autografts [8]. However, the harvesting of bone cells, skin or blood vessels requires the patient to undergo additional operations with their associated risks and for some patients they do not have suitable tissue for harvesting. Allografting or transplantation from a donor is the most effective or only available treatment for many patients. Although allografts are primarily associated with organ transplants they also include bone marrow transplants for patients suffering from various forms of haematopoietic malignancy and corneal transplants for the restoration of vision. For patients with life-threatening end-stage organ failure of the lungs, kidney, heart and liver their only option is organ transplantation. However, allograft organ transplantation is associated with numerous risks including rejection, infection and the patient's requirement of life-long immunosuppressant therapy. In 2005, 27,527 organ transplants were performed in the U.S. [9] compared with 2,880 in the UK and Republic of Ireland in 2000 (Table 1) [10] but these figures do not come close to meeting the demand [11-13] (Table 2). In the U.S. suitable liver donors were found for 1 in 4 patients requiring a transplant in 2005 [9] while only 1 in 12 patients in need of a heart transplant in the UK and Ireland received a donor organ [14]. This situation has been exacerbated by the increase in the number of patients requiring transplants which increased by 5% in the U.S. between 2004 –

2005 while over the same time period the number of organ and tissue donations has increased by 3.5% [9] resulting in less than one third of all patients requiring a transplant being found suitable donor organs [15].

An increase in the availability of donor organs for transplantation would therefore have a major impact on health and this has driven a resurgence of interest in the potential of xenograft application. Xenografting is the transplantation of tissue from one species to another. Chemically-treated xenografts, such as the porcine heart valve, have been used clinically with wide acceptance for many years. More recently acellular porcine, bovine and horse tissue harvested from a variety of sources including the subintestinal submucosa [16-18] and bladder [19] have been clinically applied for a variety of applications [20, 21]. However, the transplantation of viable xenografts runs the risk of xenozoonose and porcine endogenous retrovirus transmission to humans. Additionally, viable porcine tissue transplants are rejected in animal models thus preventing their clinical application [14, 22].

Table 1. Transplants performed in the United States in 2002 [23] and the UK and Republic of Ireland (RoI) in 2000 [24]

Organ(s) Transplanted	Number of Transplants Performed	
	U.S.	UK and RoI
Cornea	-	2,320
Kidney	14,400	1,823
Liver	5,300	709
Heart	2,200	217
Lung	1,000	98
Kidney and Pancreas	900	-
Pancreas	550	-
Intestine	104	-
Heart and Lung	31	33

Table 2. Number of patients requiring organ transplants in the U.S. in 2000 – 2001 compared with the number of transplants performed in 2002

Transplant Organ(s) Required	No. on waiting list (30-06-01) [25]	No. of transplants performed in 2002 [23] (est. of % required)	No. of patients who died while on waiting list (01-07-00 – 30-06-01) [25]
Kidney	49,860	14,400 (29)	2,837
Liver	18,089	5,300 (29)	1,799
Pancreas	979	550 (56)	23
Kidney-Pancreas	2,587	900 (35)	220
Heart	4,200	2,200 (52)	608
Lung	3,798	1,000 (26)	497
Heart-Lung	222	31 (14)	35
Intestine	170	104 (61)	24
All	79,902	24,485 (31)	6,043

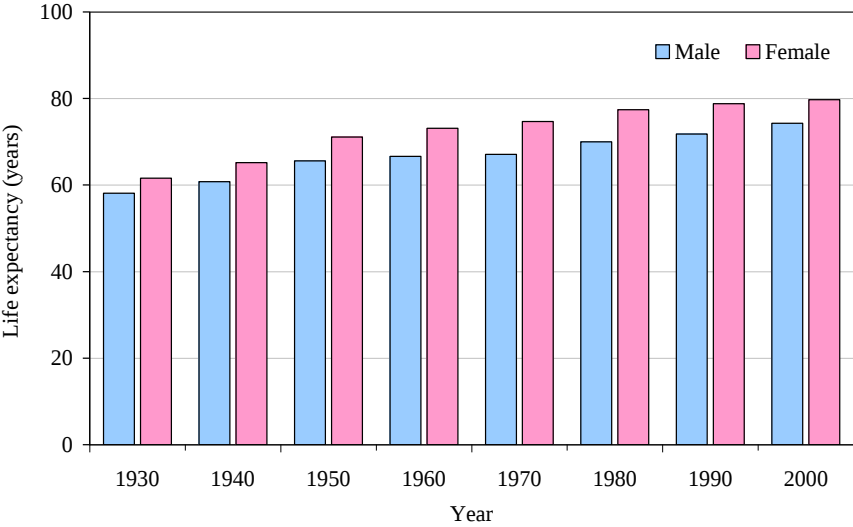


Figure 1. Changes in life expectancy for men and women in the U.S. during the 20th century [30].

Table 3. The number of operations estimated to have been performed in the U.S. in 1989 [26]

Anatomical Site	Operations per year
Skin	4,750,000
Bone	1,340,000
Cartilage	1,150,000
Tendon/Ligament	123,000
Urological	82, 000
Blood vessels	1,360,000
Pancreas	738,000

2. The increase in life expectancy has led to a greater demand for reconstructive surgery and an extended durability of implants. In 1988 over 8 million surgical procedures were performed in the US alone to treat patients suffering from organ or tissue failure [26] at an estimated cost of 400 billion \$US [27, 28] (Table 3). These figures are increasing partly due to an increase in life expectancy [29, 30] (Figure 1) but also because of a change in population dynamics *e.g.* the number of people >50 years in the U.S. was 25.7% in 1990, 27% in 1998 and is predicted to reach 32% by 2010 [31]. These changes have led to an increase in surgical interventions required to treat age-related degenerative diseases such as osteoporosis, atherosclerosis, degenerative disc disease and macular degeneration [32] (Table 4) and have also resulted in the need for implants to possess greater than 30-year survivability rates. Currently the mean lifespan of many cardiovascular prostheses is 15 years [33] (*e.g.* heart valves, bypass grafts), conventional hip replacements performed in patients less than 50 years of age have a 80% survivability rate 10 years postoperatively [34], while in the treatment of macular degeneration retinal pigment epithelial cell (RPE) transplantation has recently be explored [35].

Table 4. Comparison of the number of devices estimated to have been used in the U.S. (No date given^{*}, 2000^{*}, 2002^{*} or 2003[†])

Clinical Discipline	Application	Number of devices used per Year		
		U.S.		Globally
		Ratner <i>et al</i> (1993) ^[36]	Ratner <i>et al</i> (2004) ^[37]	
Ophthalmology	Intraocular lenses	1 400 000	2 500 000 [*]	
	Contact lenses	2 500 000		
	Retinal surgery implants	50 000		
	Prosthesis after enucleation	5 000		
Cardiovascular	Vascular grafts	350 000	300 000 [*]	
	Coronary stents		1 500 000 [*]	
	Heart valves	75 000	82 000 [*]	274 900 ^[38]
	Pacemakers	130 000	400 000 [*]	
	Cardiac assist devices			
	Artificial hearts			
Reconstructive	Breast prosthesis	100 000	250 000 [*]	
	Nose, chin	10 000		
	Penile	40 000		
Dentistry	Dental	20 000	910 000 [*]	
Orthopaedic	Hips	90 000	250 000 [*]	700 000 ^[39]
	Knees	60 000	250 000 [*]	700 000 ^[39]
	Shoulders, finger joints	50 000		55 000 ^[39]
	Bone fixation plates			

- Treatments for organ failure, such as kidney dialysis for acute renal failure and haemofiltration for acute liver failure (SeptetTM, Arbios Systems Ltd [40]), are only short-term solutions in the management of the deleterious effects of the dysfunctional organ on the patient's general health [13]. Acute renal failure affects about 200, 000 individuals in the U.S. and has a mortality rate of 55-70% even with haemodialysis support [41, 42]. Although advances in the development of non-allograft whole organ kidney transplants are being made, they are not expected to become clinical therapies in the foreseeable future. An extracorporeal kidney-assist device combines immobilised organ cells on a permeable membrane in a bioreactor and offers the advantage over conventional haemodialysis in that it aims to restore the reabsorption and endocrine functionality of the kidney [43, 44]. A temporary Renal Bio-Replacement TherapyTM developed by Dr Humes (marketed by RenaMed as RBI-01 but acquired from RenaMed Biologics, Inc. by Nephron Inc., in a purchase of its assets in 2007) successfully completed Phase II clinical trials with a 72% improvement in the 28-day survival rate of patients receiving renal bioreplacement therapy compared with conventional therapy [45,46].
- Diabetes is one of the most serious challenges in healthcare world-wide as the number of diabetic patients is predicted to increase to 220 million by 2010, a doubling of its 1994 prevalence [47]. Recently developed pharmacological therapies show improved control of blood glucose levels in the treatment of Type II diabetics (*e.g.* Liraglutide (NN2211) [48, 49]) but Type I diabetics are dependent on insulin injection which results in fluctuations in their physiological blood glucose levels. Elevated blood glucose levels triggers the onset of secondary microvascular and neurologic complications such as cardiovascular disease, glomerulonephritis and

proliferative diabetic retinopathy (PDR) [50]. PDR is a major cause of blindness globally affecting 4 % of the world's population (with a projected increase to 5.4% by 2025) [51, 52]. It affects 4.1 million adults over 40 years in the United State (predicted to increase to 6.1 million persons by 2020) with 300 000 of these adults expected to become legally blind as a consequence of PDR within 3 years [53]. Recent approaches to PDR management include oral administration of protein kinase C (PKC) inhibitors (PKC β [54,55], candesartan, cilexetil and octreotide) [56], which are under Phase III clinical trials [57], anti-VEGF [58-61] and sustained-release steroid implants (Retisert [62]). Although the latter approach reduces retinopathy its clinical applicability is questionable as it is associated with cataract formation and a 33% incidence of glaucoma [63]. The intense clinical management of glycaemia levels however reduces the risk of microvascular and neurologic complications of Type 1 diabetes. Normoglycaemic levels can be achieved by either increasing the number of daily insulin injections or by treatment with an external insulin pump with dosages being adjusted by self-monitoring glucose measurements [64]. There is, therefore, a demand for approaches to Type I management that control glycaemic levels without relying on patient monitoring.

5. The presence of long-term, indwelling implants predisposes the patient to the lifelong risk of infection and an acquired hypersensitivity [65, 66] to the implanted material necessitating removal of the implant.
6. In paediatric cases the inability of prosthetic substitutes to grow has also limited their widespread clinical application. Additionally, growth of the individual is usually impaired following organ transplantation as a side-effect of steroids used as immunosuppressants [67].

There is, therefore, a need for restorative device designs that improve implant durability and alternatives to augment the currently available clinical therapies. The two main approaches being taken to address these needs are: the development of reconstructive materials with enhanced biological integration and the development of materials designed to aid tissue regeneration.

CLINICAL APPROACHES

Reparative Reconstructive Surgery

a. Biomaterials in reparative reconstructive surgery

The use of biomaterials (or medical devices or prostheses) in reparative and reconstructive surgery in medicine and dentistry to treat, augment or replace dysfunctional tissue is not a new approach and in fact can be dated back to before 800 BC (Table 5). However, with improvements in aseptic surgical techniques and technological advances in biomaterials science there are now more than 2,700 different kinds of medical devices available [68] with an annual global market value exceeding 36 billion \$US [69] with a predicted growth rate of 12% per year [70] (Table 6). Prominent applications of biomaterials include (Figure 2): orthopaedics [39] (*e.g.* hip and knee joint replacements [66, 71-75], bone

cements [76, 77], bone fillers [78-80], fracture fixation plates [81-83], and artificial tendons and ligaments [84-87]), cardiovascular [38] (*e.g.* vascular grafts [6, 88-91], heart valves [91], pacemakers [92], stents [93]), ophthalmics [94,95] (*e.g.* corneal implants and artificial corneas [96, 97] and intraocular lenses [98, 99]), dental implants [100] and cements [101-104], cochlear implants [105], tissue adhesives and sealants [106], drug-delivery systems [107] and sutures [88, 108].

Table 5. A brief historical overview of some of the major achievements in the application of materials in medicine and dentistry

Year	Development	Reference
800 BC	Egyptians used linen sutures and strips soaked in natural adhesives to draw wound edges together	[109]
600 BC	Etruscan gold bridge work	[110]
1400's	American Indians used horsehair, cotton and thin strips of leather in the treatment of wounds	[109]
1775	Use of wires of brass, silver and gold in the treatment of bone fractures	[111]
1849	Introduction of the use of percutaneous metal hooks to stabilise fractures	[112]
1895	Bone plates were developed	[113]
1937	Poly(methylmethacrylate) (PMMA) was first used in dentistry	[114]
1950's	Alloys such as stainless steel, cobalt-based alloys and titanium were used in orthopaedics	[39]
1950's	First PMMA cemented hip replacement using a stainless steel femoral stem and UHMWPE acetabulum	[73, 74]
1952	Dacron [®] arterial prostheses became commercially available	[115]
1960's	Development of first bioresorbable sutures Dexon [®]	[116]
1961	Contact lenses developed by Wichterle	[117]
1968	Development of a tanned porcine aortic heart valve mounted on Dacron [®] fabric coated stents	[118]
1970's	Microporous expanded polytetrafluoroethylene (ePTFE) vascular grafts introduced	[119]
1970	Use of collagen in full thickness wound healing in animal models	[120]
1970	Alumina was used in hip replacement	[39]
1972	Bioactive glasses with bone-bonding ability were developed	[121]
1973	Suturing of lacerated tendons	[122]
1974	Development of composite degradable sutures of Poly(glycolic acid) and Poly(lactic acid)	[123]
1975	First glass ionomer cement, ASPA, used in dentistry	[124]
1981	Polydioxanone was developed as a suturing material	[125,126]
1983	Porous calcium phosphate was used in medical and dental applications	[127]
1985	Bioglass [®] Ossicular Reconstruction Prosthesis (MEP [®]) for ossicle replacement	[121]
	Resin-modified glass ionomer cements	[128]
1994	Particulate Bioglass [®] : NovaBone [®] approved as a bone void filler, Perioglas [™] for periodontal disease	[121,129]
1994	FDA approval of coronary artery stenting	[130]
1994	First soft biomaterial for IOLs introduced by Alcon Laboratories Inc (Acrysof IOL)	[131]
1995	Daily disposable lenses available on the market	[132]
1995	Haptex [™] licensed in the UK as a middle ear bone ossicle replacement	[133]
2000	Orthovita receives FDA Clearance for VITOSS Scaffold the First Engineered 90% Porous Beta-Tricalcium Phosphate	[134]
2001	Newer generation of soft silicone foldable IOLs <i>e.g.</i> Collamer [®] IOL, Crytalens [®] AT-45	[135,136]
2003	FDA approval of first drug-eluting coronary artery stent	[137,138]
	Approval of Crytalens [®] AT-45 Accommodating Posterior Chamber Intraocular Lens (IOL) used to correct the visual impairment of aphakia (absence of the natural eye lens) after cataract surgery	[139]
2004	Bioglass [®] particulate approved for treatment of tooth hypersensitivity	[121]
2007	Angiotech Pharmaceuticals Inc received clearance from the FDA to market Rex Medical LP's chronic dialysis catheter	[140]

Hard Tissue

Dental Implants

Metals:

Gold, Platinum, Palladium and Stainless steel

Ceramics:

Alumina, Calcium phosphate, glass and glass ceramics, Carbon

Polymers:

UHMWPE, PTFE, PMMA

Hip Joint

Acetabular cup:

Ultra High-Molecular-Weight Polyethylene (UHMWPE)

Articulating ball (load-bearing):

Alumina, Zirconia, cobalt-based alloys

Surface coating of load bearing implant:

Hydroxyapatite, Bioactive glasses and glass-ceramics

Femoral stem:

Titanium, 316 L SS

Ear

Outer:

Poly(dimethyl siloxane)

Middle:

Cervital glass ceramic, Bioglass[®]

Chin

Poly(dimethyl siloxane)

Finger Joints

Poly(dimethyl siloxane), UHMWPE

Knee

UHMWPE

Hydrogels

316L SS

Bone

Load bearing materials:

Alumina, Titanium, Stainless Steel

Coating of load bearing:

Hydroxyapatite, Calcium Phosphate

Fixation devices:

PLA-carbon fibres, PMMA

Soft Tissue

Eye

Contact lens:

PHEMA and HEMA copolymers

Retinal detachment surgery:

Silicone oil, Perfluorodecalin, Silicone rubber, PMMA

Heart Valves

Occluder:

Silastic

Leaflets:

UHMPE, pyrolic carbon

Struts:

Titanium, Cobalt-chrome alloy, pyrolic carbon

Sewing ring:

Silicone under a knitted composite of Teflon and polypropylene; Teflon[®]/Dacron[®], PTFE fabric over silicone rubber filler

Arterial Prostheses (diameter >5mm)

Poly(tetrafluoroethylene) (ePTFE or Teflon[®])

Dacron[®]

Polyurethanes

Nylon

Arteries
Polyester, Polyanhydride, PVC

Venous
Polyester, polyanhydride, PVC

Tendons and Ligaments

PLA/Carbon fibre

ePTFE

PET

UHMWPE

Figure 2. Some examples of the use of implantable biomaterials in medicine and dentistry [88, 94, 141-147].

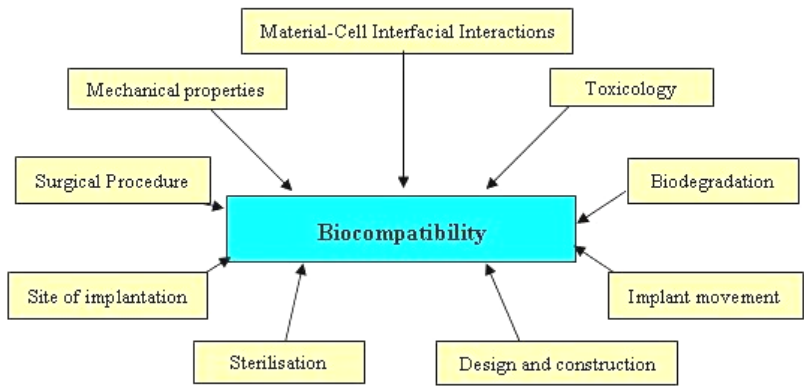


Figure 3. Diagrammatic representation of factors that influence the functional biocompatibility of an implantable device.

Table 6. Major clinical speciality markets for biomaterials

Application	Market (Billion U.S.\$)			Projected Increase (%)
	Europe	U.S.	Global	
Orthopaedic (2000) [69]	3.2	9.16	15.8	
Orthopaedic (2002) [39]	-	-	14	7 – 9 [39]
Fracture management devices (2000) [39]	-	-	1.5	
Hip replacement (2002) [39]	-	-	2.5	
Knee replacement (2002) [39]	-	-	2.5	
Cardiovascular (2000) [69]	1.8	5.4	8.1	
Vascular Graft (2000) [119]	-	-	0.2	
Drug Delivery (2000) [69]	1.7	2.1	6.3	
Wound Care (2000) [69]	1.9	1.8	4.7	

b. Factors governing the clinical performance of implantable biomaterials

Reconstructive surgery relies on the excision of damaged tissue and its replacement by a non-viable, biocompatible biomaterial substitute or prosthesis. An implanted material’s biocompatibility, defined as ‘the ability of a biomaterial to perform with an appropriate host response in a specific application’ [148], is pivotal to its clinical success. Numerous factors influence a material’s biocompatibility as illustrated in Figure 3. The relative importance of each of these factors is dependent upon the application but is primarily influenced by the material-tissue response, contact duration, anatomical site and functional requirements.

For a material to be biocompatible, it must:

1. Meet the functional demands of its application *e.g.* be capable of maintaining a load over a few months if it is to be used as a bone plate to immobilise a fracture [149]; reduce water evaporation if it is to be applied as a wound dressing [150,151]; have an optimal refractive index if it is to be used as a vitreous replacement in the eye [152].

2. Elicit an appropriate host response. This allows for complete inertness, should that be desirable or attainable, but it equally allows for specific biological activity that produces a beneficial effect for the recipient. The host response is the reaction of the tissue to the implant, which controls the physiological performance of the patient following placement of the implant and is itself controlled by the characteristics of the material especially by the material's chemical stability at the anatomical site.
3. There is also the need to consider the site of application (*i.e.* specific applications). The biocompatibility characteristics required of a material are related not only to the functional requirements but are also governed by the local physiological environment. The latter varies anatomically and there is therefore no such thing as a biocompatible material *per se* (*e.g.* the properties of an intraocular lens are quite different from those required of a vascular prostheses).

In investigating the clinical applicability of a material the assessment of its mechanical properties, its wear and degradation (originating from both mechanical and biochemical sources) and the material-tissue interfacial response, at the intended site of implantation, provide indications of deficiencies in biocompatibility. Inappropriate materials' selection has resulted in gross patient disfigurement and fatalities. For example, in the mid-80's 25,000 patients had a temporomandibular joint (TMJ) device, composed of a carbon-alumina porous composite (Proplast[®]) and a PTFE film, implanted. Following implantation, all of these devices failed due to the build-up of PTFE fragments because of frictional wear debris. The wear debris triggered a giant cell foreign body response causing severe inflammation and extensive bone erosion [153]. For all of these patients re-operation to remove the implant was necessary. Nearly all of the patients were subsequently left unable to chew and were in constant pain whilst other patients also suffered severe facial deformities. The use of zirconia had been advocated for femoral head replacement [154] however the quality of zirconia is highly dependent on the precise manufacturing process used. A change in the manufacturing process in 1988 led to 1 in 3 devices failing [155] as a result of post-implantation grain pull-out increasing the surface roughness 20-fold and by the accelerated transformation of the zirconia from the tetragonal to monoclinic phase in the central area of the head resulting in fracture [156, 157]. Another example of the inappropriate use of a material, due to leachate release, was the application of glass ionomer cement (GIC) in the repair of a skull-base defect following cranial surgery. GICs are used as bone cements in other non-loading applications but the proximal placement of the aluminium-based cement with brain tissue resulted in two fatal cases of post-otoneurosurgery aluminium encephalopathy due the blockage of nerve conduction by released aluminium [158]. These examples show the need for careful consideration of the tissue-material interactions in their entirety for each application.

From the perspective of mechanical compatibility polymeric materials have historically been favoured for soft-tissue replacement and metals or ceramics for load-bearing hard-tissue replacement because these classes of materials have physical properties similar to that of the tissues they are replacing as demonstrated in Table 7. However, the indicated functional properties of tissues are based on static measurements, which although a useful guideline in material development, do not indicate the influence of cyclic loading and shear. Functional material assessment must therefore also reflect long-term biomechanical performance.

Table 7. Comparison of the mechanical properties of some selected tissues and materials used in specific clinical applications (L = Longitudinal, Trans = Transverse, Circ = Circumferential, C = compression, T = tension)

Tissue Type/ Material	UTS ¹ (MPa)	Elongation to break (%)	Young's Modulus (GPa)	Clinical Application
Aortic Heart Valve (Radial) [159]	0.045	15.3		
Aortic Heart Valve (Circ) [159, 160]	2 - 4.5	10 - 18	41 - 64	
Human Aorta (L) [159]	0.07	77		
Human Aorta (Trans) [159]	1.1	81		
Artery [160]	1 - 1.6	0.8 - 1.1	0.03 - 3	
Dacron [®]	<40 ^[161] , 59 - 72 ^[162]	50 - 300 ^[162]	2.8 - 4 ^[162]	Arterial graft; Tendon and ligament
Teflon (PTFE)	14 - 34 ^[161]	200 - 400 ^[162]	0.4 ^[161]	Arterial graft; Tendon and ligament; Catheter
Elastic Cartilage	3	30	15	
Articular Cartilage [159]	3.4		10 - 21	
Skin [159, 160]	6.2 - 14	78 - 140	23 - 44	
Tendon [160]	59 - 69	8 - 9	966	
Achilles Ankle Tendon [159]	24 - 61	24 - 50		
Human Enamel (Molars)	10 ^[159]		50 ^[163]	
Human Dentin (Molars)	34.5 - 52 ^[159]		18 ^[163]	
Glass Ionomer Cement [163]	170 - 260(C)			Dental
Tibia Fascia [159]	10 - 18			
Femoral Bone (L) [75]	130	3	17(T)	
Femoral Bone (Tangential) [75]	60	1	12	
Femoral Bone [160]	120	1.4	17	
Cortical Bone (L)	133(T) ^[163, 164] 130 - 180(C) ^[165]	3.1	10.9 - 29.2 ^[164] 7 - 30	
Cortical Bone (Tangential) [159, 164]	52	0.7		
Spongy bone [75]	2	2.5	0.1	
PMMA [*] (Solid)	35-50, 65(T) ^[75]	0.5, 5 ^[75] 2 - 10 ^[162]	3 ^[75]	Orthopaedic; Intraocular lens
PMMA Bone Cement [75]	30(T)	3	2	Bone cement
Glass Ceramic [75]	200	<0.1 ^b	200	Bone cement
Bioglass [165]	1000(C)		~75	Spinal fusion
Alumina	260(T) ^[75] 4000(C) ^[165]	<0.1 ^{b[75]}	400 ^[75] 380 ^[165]	Femoral head
Dense Hydroxyapatite [75]	200	<0.1 ^b	120	Coating on femoral stem, Bony defect repair
Zirconia	2000(C) ^[165]		150 - 200 ^[165]	Femoral Head
Titanium Grade 4 [166]	760		110	Dental implant for tooth fixation

¹ UTS = Ultimate Tensile Strength

^{*} PMMA = poly(methylmethacrylate)

^b estimated values

Table 7. (Continued)

Tissue Type/ Material	UTS ² (MPa)	Elongation to break (%)	Young's Modulus (GPa)	Clinical Application
Ti6Al4V	860 – 990(T) ^[75, 163, 166]	10- 14 ^[75, 167]	110 ^[75]	Femoral stem; knee; Dental implant for tooth fixation
Stainless Steel 316L	1000(T) ^[75, 163]	9 ^[75]	200 ^[75]	Femoral stem, Bone plate for fracture fixation
UHMWPE*	7.6, 30(T) ^[163]	150, 200 ^[75]	1 ^[75]	Cemented acetabular cup
Polysulphone [75]	70	50	2.5	Orthopaedic bone plates, screws, intramedullary nails
Silicone Rubber [75]	6	350	<0.01	Orthopaedic; Catheter; Intraocular lens
PEEK	90 ^[161]		3.6 ^[161]	
Polyurethane	1 – 69 ^[161]	600 – 720 ^[166]	0.07 – 6.9 ^[161]	Arterial graft; Artificial heart
D,L-PLA (107 – 550 x 10 ³) [162]	29 – 35	5 - 6	1.9 – 2.4	Bone Plate

From a cellular perspective implanted materials from non-biological sources are not attacked by the immune system and have therefore been classified according to the histology at the biomaterial-tissue interface following implantation as inert, resorbable or bioactive [168] (Table 8). Up until the late 1970's it was considered essential for a material to be inert (or pseudoinert) in order to achieve long-term clinical patency. The implantation of an inert biomaterial perturbs the normal wound healing response (Figure 4) and initiates a sequence of events equivalent to a foreign-body reaction (with the exception of titanium which becomes closely approximated to 'nearly normal' host tissue with no intervening fibrous capsule). The sequence of events starts with an acute inflammatory response (Figure 5) and leads, in some cases, to a chronic inflammatory response and/or granulation tissue development, a foreign-body reaction (a special form of non-specific inflammation) and fibrous encapsulation [169]. Fibrous encapsulation walls off the implant from the surrounding tissue by the formation of a fibrous capsule that is formed in the same manner as scar tissue in the normal wound healing response *e.g.*, PMMA bone cement [170-172] and silicone breast implants [173]. The duration and intensity of each of these phases is strongly influenced by numerous factors [174, 175]: the primary chemical structure and composition [176], the surface free energy [174, 177-179] and charge, the implant size, shape [180], porosity and roughness [174, 176] and by the invasiveness of the implantation procedure. The capsule is maintained due to the continued presence of the implant and the capsule thickness is influenced by factors including [174]: motion between tissue and implant with thickness increasing with relative motion [170], chemical activity of material (*e.g.* corroding metals or leaching of polymers where the thickness of the capsule is proportional to the rate of released chemical irritant [181]), the presence of electrical current (*e.g.* the ends of the stimulating electrodes in a pacemaker with the thickness of the capsule being proportional to the current density), and the shape of the

² UTS = Ultimate Tensile Strength

* UHMWPE = Ultra high molecular weight polyethylene ($> 2 \times 10^6$ g/mole)

implant (e.g. edges and sharp surface features) also increase capsule thickness [180]. In all cases if the implant is removed the capsule may collapse into a residual scar or be completely remodelled.

Wound repair following the implantation of a resorbable material (Table 8) is influenced by the rate and mode of resorption and by the tolerance of the local tissue to the degradation products. The host tissue may therefore treat the material as a component of the 'normal' tissue and passively resorb the material or it may be walled-off in a manner analogous to the inert materials. In the latter case following resorption of the material a collapsed scar forms at the implant site that subsequently remodels.

A. Inflammatory Phase

Cellular/Humoral response

1. Damage to tissue and surrounding capillaries results in the release of blood into the wound cavity
 - o Activation of the clotting system and/or thrombosis
 - o Coagulation factor XII contacts collagen, foreign proteins, or a foreign material
2. Fibrous clot formed by platelets and fibrinogen at the site of injury.
 - o Local capillaries dilate and the permeability of the vessel endothelium increases
 - o Increased blood flow to region
 - o Redness due to increased concentration of red blood cells
 - o Outflow of plasma to surrounding tissues leads to swelling and pain

Cellular Invasion

- o Starts within minutes to hours of injury and results in the migration of inflammatory cells to the wound site
- o Neutrophils move into surrounding tissue and phagocytose small particles (0.1 - 1 μm average size) or fragments of tissue or foreign material. Phagocytosis of larger particles begins later by macrophages and foreign body giant cells (FBGC's), while particles greater than 50 μm do not initiate a reaction greater than the bulk material. This process clears particles (dead tissue and foreign material) away from the site
- o Inflammation is accompanied by four classical symptoms: redness, swelling, pain and heat. The magnitude of these symptoms is indicative of the degree of inflammatory response
- o If tissue injury is extensive or the wound contains irritants or bacteria, substantial tissue damage may occur due to the extracellular release of collagenase

B. The Proliferative or Regeneration Phase

3. Capillaries at site begin to form buds within the fibrous clot
 - o Granulation tissue formed: characterised by new capillary formation and an increase in fibroblast proliferation
 - o Formation of new connective tissue: collagen and GAGs are synthesised by fibroblasts
4. Capillaries grow through clot and anastomose re-establishing blood supply

C. Repair and Remodelling

5. Scar tissue forms and begins to remodel
 - o Forms scar tissue at site which acts as scaffold for cellular reconstruction
 - o Myofibroblasts activity leads to wound contraction
 - o As granulation tissue is remodelled there is a decrease in the number of fibroblasts and vessels in the wound.
 - o Collagen is remodelled into large-diameter fibres and the concentration of proteoglycans changes.
 - o In skin Type I collagen fibres align to form a scar that is approximately parallel to the surface of the skin.

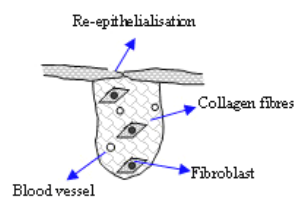
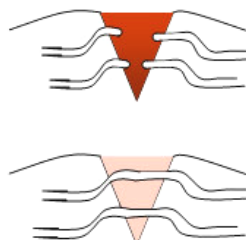
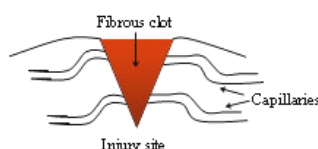
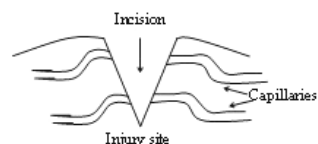


Figure 4. Overview of events following injury through the full thickness of skin.

Table 8. Examples of the application of pseudo-inert, bioresorbable and bioactive materials (adapted from Williams [182])

Material Classification	Material		Application
Inert	Metals	Titanium alloys	Femoral stem and head[39, 167]
		Stainless steel	Orthopaedics[39, 75, 167], heart valves[37]
	Ceramics	Alumina	Femoral head, articulating joints[183]
		Zirconia	Femoral head[154, 167, 184]
	Polymers	Silicone rubber	Scleral buckles[142], Catheter[185]
		ePTFE	Bypass grafts[6]
		PMMA	Bone cement[186]
Resorbable	Ceramics	UHMWPE	Articulating joints[73, 74]
		Calcium Sulphate	Bone graft*[79, 80]
	Polymers	Tricalcium Phosphate (TCP)	Bone graft*[187, 188]
		Poly(L-lactic acid) (PLA)	Suture[189], Bone fixation[190-192]
		Poly(glycolic) acid (PGA)	Sutures[189, 193], Bone fixation*, Drug delivery
		Poly(D,L-lactic acid)	Intramedullary plug[194]
		PGA/PLA composite	Sutures[195], Bone fixation
		Poly(α -cyanoacrylate)	Bioadhesives, drug delivery matrices
		Poly(ϵ -caprolactone)	Drug delivery*, orthopaedic applications
		Poly(orthoesters)	Drug delivery[186, 197]
		Polydioxanone	Sutures, Suture clip, bone pin*
		PHB, PHV and their copolymers ³	Drug delivery, sutures, vascular grafts[198]
		Hyaluronic acid esters	Wound healing[199]
		Collagen	Soft-tissue augmentation[200] <i>e.g.</i> urinary incontinence*
		Fibrin	Bioadhesive*[201-207] Drug delivery[208-212]
Bioactive	Inorganic	Bioglass [®]	Middle ear[121], synthetic bone graft[121]
		Hydroxyapatite	Coating of bone implanted devices*[186]
		Glass Ceramics	Dentistry[213]
	Polymers	Galactosylated PVDF ⁴ membrane	Promotes adhesion of hepatocytes[214]
		ePTFE + immobilised VEGF	Promotes adhesion of endothelial cells[215]
		Hydrogels + coupled RGD peptides	Promotes healing of diabetic ulcers[216, 217]
		ePTFE + arg-gly-asp(RGD)	Stimulation of endothelial adhesion[218]
		ePTFE + immobilised heparin	Improved haemocompatibility of bypass graft[219]

³ PHB = Poly(hydroxybutyrate) and PHV = Poly(hydroxyvalerate)⁴ PVDF = poly(vinylidene difluoride)

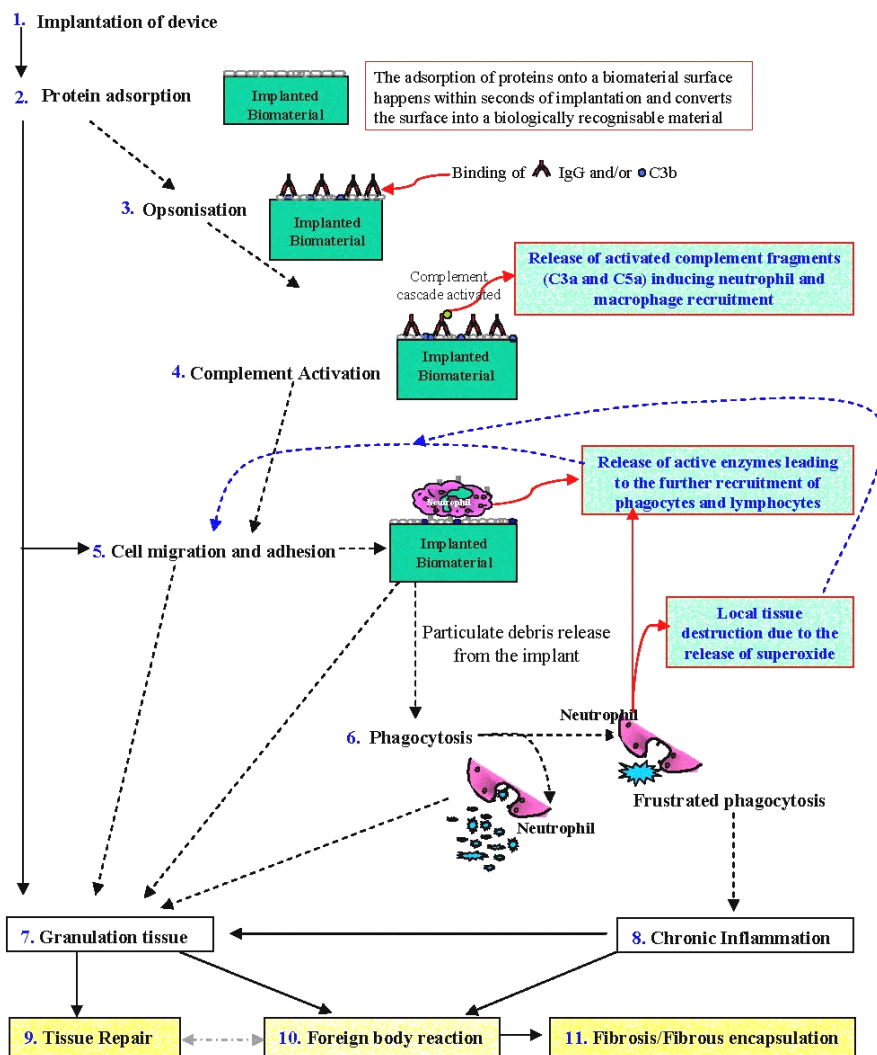


Figure 5. Schematic representation of events leading to fibrous capsule formation following the implantation of a biomaterial⁵[1, 173, 174, 220-225].

The implantation of a medical device results in the release or activation of inflammatory mediators by the injured tissues (histamine, kinins, prostaglandins, leukotrienes, IL-1, IL-6 *etc*) and platelet adherence and aggregation on the endothelial surface of damaged vascular tissue releasing serotonin and fibrinogen. The adjacent blood vessels dilate and the permeability of the capillary walls increases enabling proteins and cells to move to the injury site. This exudation of proteins produces a differential osmotic pressure between the blood and the interstitial space in the injured tissue resulting in water entering the tissue.

The clotting proteins from the blood diffuse into the interstitial spaces and form clots in the injured tissues and blood vessels. The clot effectively walls off the injured site from the body by laying down a fibrillar matrix, containing fibrin (on which fibrinogen, thrombospondin and platelet granules are bound), complement proteins, activated platelets (which release platelet derived growth factor (PDGF), transforming growth factor- β (TGF- β) (which increases ECM synthesis), platelet-derived endothelial growth factor (pdEGF) and platelet factor α , neutrophils and endothelial cells. The clot is stabilised by the crosslinking of fibrin by Factor XIIIa providing a scaffold for tissue repair.

During the 1980's numerous studies evaluating the material-tissue interface revealed that increased implant survivability was achieved when there was co-operative interaction between the device and the local tissue [226, 227]. The recognition of the benefits of biological interaction has transformed the biomaterials' field over the last 20 years. Bioactive materials are designed to elicit specific, beneficial responses that may be brought about by encouraging tissue ingrowth or adhesion. Tissue ingrowth is a desired response for many

Implants become exposed to this complex mixture of tissue and plasma proteins which selectively adsorb onto the material's surface within seconds - minutes following implantation. The composition of the adsorbed protein layer is influenced by the material's surface chemistry and topography. It is this layer of adsorbed proteins, and lipids, which modulates cell adhesion at the material-tissue interface and triggers the biological cascades. In general, vitronectin adsorption promotes endothelial cell adhesion and a relatively quiescent wound healing response while fibrinogen adsorption promotes platelet (CD41), neutrophil and macrophage adhesion and therefore a far more aggressive local environment.

Opsonisation: Opsonins, such as Ig G and complement C3b, may adsorb onto the material surface which bind to receptor ligands on neutrophils and macrophages.

Complement activation, in the presence of an implanted device, generally occurs by the alternative pathway although there is evidence that the classical pathway can also contribute presumably subsequent to IgG binding. The binding of C3b to a material's surface triggers the complement cascade with the production of the soluble chemoattractants C3a and C5a which induce phagocyte activation and recruitment to the injury site.

Phagocytic cell migration across the endothelium to the site of tissue injury is initiated by their binding to cell adhesion molecules expressed on activated endothelial cells (*e.g.* ICAM-1, VCAM-1 and MAdCAM-1 and E- and P-selectin), whilst activation is triggered by chemotactic molecules (*e.g.* C5a, leukotriene-B₄, fibrin peptide B and thrombin) and by chemokines (IL-8 and MCP-1). The interaction of E-selectin on the endothelial cells with CD15 on leucocytes in the presence of additional chemoactive molecules results in the up-regulation of leukocyte adhesion receptors, (*e.g.* LFA-1 $\alpha_1\beta_2$ and VLA- $\alpha_4\beta_1$ and L- selectin), enabling the leucocytes to bind to ICAM-1 expressed by endothelial cells. Once the leucocytes have crossed the endothelium they interact with the ECM via α_1 -integrins or VLA receptors and are 'guided' to the site of injury by chemoattractants such as C5a. The leukocyte cell membrane receptors interact with proteins and other ligands that have adsorbed onto the material surface from the surrounding biofluids and it is this interaction that modulates the cell behaviour at the implant site.

Phagocytosis is induced when there is tissue debris and/or particulate debris from the material at the inflammatory site. Neutrophils phagocytose small particles (0.1 - 1 μ m average size), fragments of tissue or foreign material while macrophages and foreign body giant cells (FBGCs) phagocytose larger particles (< 20 μ m). Particles greater than 50 μ m do not initiate a reaction greater than the bulk material *i.e.* PMMA particles are generally encircled by a layer of FBGCs or encapsulated in a fibrous coat in the same manner as the bulk material. The phagocytic cells accordingly 'clean up' the implantation site, which is facilitated when the material is coated with opsonins. An exception to this is frustrated phagocytosis which results in the extracellular release of enzymes from activated neutrophils and macrophages that may cause additional tissue injury as seen with particulate-induced osteolysis in the presence of UHMWPE particulate debris in a THR.

Secretion of fibronectin by macrophages and fibroblasts promotes the cytokine-directed migration of endothelial cells, myofibroblasts and lymphocytes into the wound site. The ECM of granulation tissue is primarily composed of fibronectin, hyaluronic acid and Type III collagen and is characterised by new capillary formation accompanied by an increase in fibroblast proliferation.

The chronic inflammatory response delays healing in response to a prolonged chemical or physical irritation at the material-tissue interface. In general, chronic inflammation is characterised by the presence of macrophages, monocytes, lymphocytes, the proliferation of new blood vessels and the synthesis of collagen and glycosaminoglycans by fibroblasts and is influenced by the concentration of cytokines such as IFN- γ and TNF which activate macrophages and IL-10, IL-4 and IL-13 that inhibit macrophage activation.

Tissue repair occurs by regeneration or replacement (with the formation of scar tissue) which of these processes dominates depends on the tissues involved and the nature and extent of the wound.

The foreign body reaction is composed of foreign body giant cells (FBGCs) (formed by the fusion of monocytes/macrophages) and components of the granulation tissue. FBGCs are formed in the presence of particulate debris at the surface of materials with a high surface area to volume ratio, such as fabrics, and when the adsorbed protein coat contains phagocyte adhesion proteins (*e.g.* IgG, C3b, vitronectin *etc.*).

Some continued inflammatory activity occurs at the implant-tissue interface as the fibrous layer formation progresses to encapsulate the material predominantly composed of collagen Type III. The thickness of this layer is influenced by the chemical activity of the implant and mechanical factors such as implant micromotion.

implants and has been seen to occur with a wide variety of materials, including metals, ceramics, and polymers. Cellular elements must adhere to the graft surface for ingrowth to occur which is affected not only by the mechanical stability of the implant-tissue interface but also by the surface chemistry, topography and bulk morphology of the implant. For example, tissue in-growth occurs in interconnected porous materials but the nature of the in-growing tissue is dependent on the minimum size of the interconnections between pores. For example, soft tissue will be found in pores with interconnections as small as 1 - 5 μm , mineralized tissue begins to form between pores of 50 and 150 μm while osteonal bone grows into pores of $\geq 250 \mu\text{m}$ [228]. An alternative approach, tissue adhesion, is encouraged when there is a close approximation of the tissue and implant. Tissue adhesion occurs by two mechanisms: tissue integration and through surface active responses. In tissue integration cells are encouraged to bind onto proteins adsorbed to the implant surface (Figure 6) [229] while adhesion, induced by surface active responses, is accompanied by a chemical alteration of the implant surface with true tissue bonding resulting in a continuous gradation of properties (both structural and compositional) across the implant-tissue interface.

c. Approaches to improving device performance in reconstructive surgery

Many of the developed 2nd generation biomaterials are bioactive materials, or pseudo-inert materials with a bioactive coating, in which improvements in their long-term patency have been attained by encouraging integration between the material and local tissue. For instance:

1. A total hip replacement (THR) is comprised of a femoral stem, femoral head and an acetabular cup [75]. Survival rates for conventional total hip replacements in patients over 65 years at the time of implantation show 80% patency 20 years postoperatively [230]. However, the 15-year patency rates for patients < 50 and < 40 years at the time of implantation are 60 and 54 %, respectively [231]. The significant reduction in the survivability rates in younger more active patients is primarily associated with bone resorption at the implant interface. Other factors influencing patency include prior history of hip fracture and revision with prosthetic femoral stem replacement procedures only being able to be performed twice on the same patient [232] as the femoral bone is weakened by the implantation procedure itself and there are significant problems encountered in removing prosthetic acetabular cups without damaging the pelvic girdle.

In all patients implant survivability is affected by stress-shielding [233], the stability of the fixation of the femoral stem and the abrasive wear resistance of the femoral head and acetabular cup. Mechanical stimulation is necessary for healthy bone maintenance but because of the modulus mismatch, between the prosthetic stem and femoral bone, tissue proximal to the prosthetic stem is stress-shielded and resorbed. This leads to aseptic stem loosening which further aggravates bone tissue destruction and accounts for 79 – 82 % of THR failures [234]. Recent attempts to resolve this modulus mismatch have addressed the processing of titanium alloys [235]. The renewed interest in titanium alloys has also been driven by the significant number of patients becoming hypersensitive to stainless steel and cobalt-base orthopaedic joint replacement components [236], and due to concerns regarding systemic toxicology

and metal implant debris-induced tumourgenesis (*i.e.*, Cr, Co, Ni are carcinogenic in rodents [237]).

Avenues taken to improve the long-term performance of THR's include:

Micro-patterning of the femoral stem to promote cell adhesion [238, 239] and the development of bioactive coatings [240] and cements [241, 242]. These latter approaches have significantly contributed to improvements in patency rates (Table 9) due to improvements in tissue integration and adhesion of the implant with the bone tissue.

Abrasive wear of UHMWPE acetabular cups is an additional significant cause of implant failure as it results in UHMWPE particulate-induced osteolysis necessitating removal of the prosthetic acetabulum. It is hoped that the implementation of changes in the manufacturing of UHMWPE acetabular cups, aimed at reducing free radical formation, will extend clinical survivability [243, 244].

Alternative prosthetic acetabular cup designs [245] have received FDA approval *e.g.*, a highly crosslinked polyethylene-on-metal (approved 1997) and an alumina-on-alumina (approved 2000). Concerns raised over the 0.026% failure rates due to liner fractures with 1st generation alumina cup designs have now been reduced to 0.004% with the 3rd generation designs [246], while the coupling of an alumina cup with an alumina liner shows 50 – 200 times less wear, compared with UHMWPE on cobalt chrome or UHMWPE on alumina ceramic [246] and show 5-year patency rates of 97.4%.

2. Improvements in the interfacial bonding, aesthetics, fracture toughness and flexural strength of posterior dental fillings are also being sought. Currently materials such as composites and resin-modified glass ionomer cements (RMGICs) [248-251] have been advocated in place of amalgams but shrinkage of resin-modified GICs away from the tooth-material interface results in secondary caries [252]. Although conventional GICs show good interfacial bonding [253, 254] their fracture toughness and flexural strength are insufficient for use in posterior Class I and II restorations [255]. New approaches to improving the mechanical properties of conventional GICs include ultrasonic setting [256] and the use of ceramic fillers [257, 258].
3. In other areas of biomaterial application, the exploitation of the ever-increasing understanding of the cell biology and biochemistry of the biomaterial-tissue interface is facilitating the production of advanced bioactive materials. This is reflected by the increase in the worldwide global market for bioactive materials *i.e.* \$377.7 million in 2004, \$431.4 million in 2005 and an estimated \$473.9 million in 2006 [259]. Bioactive ceramics and glass-ceramics designed for orthopaedic application (*e.g.*, hydroxyapatite and Bioglass[®], apatite/wollastonite (A-W)) have to date had limited clinical application due to their poor mechanical properties [260, 261] resulting in the focus of orthopaedic development being aimed at improving the chemical bonding of bioactive cements with the bone tissue, the production of aluminium-free bone cements for spinal and cranial surgical applications and glass ceramics.

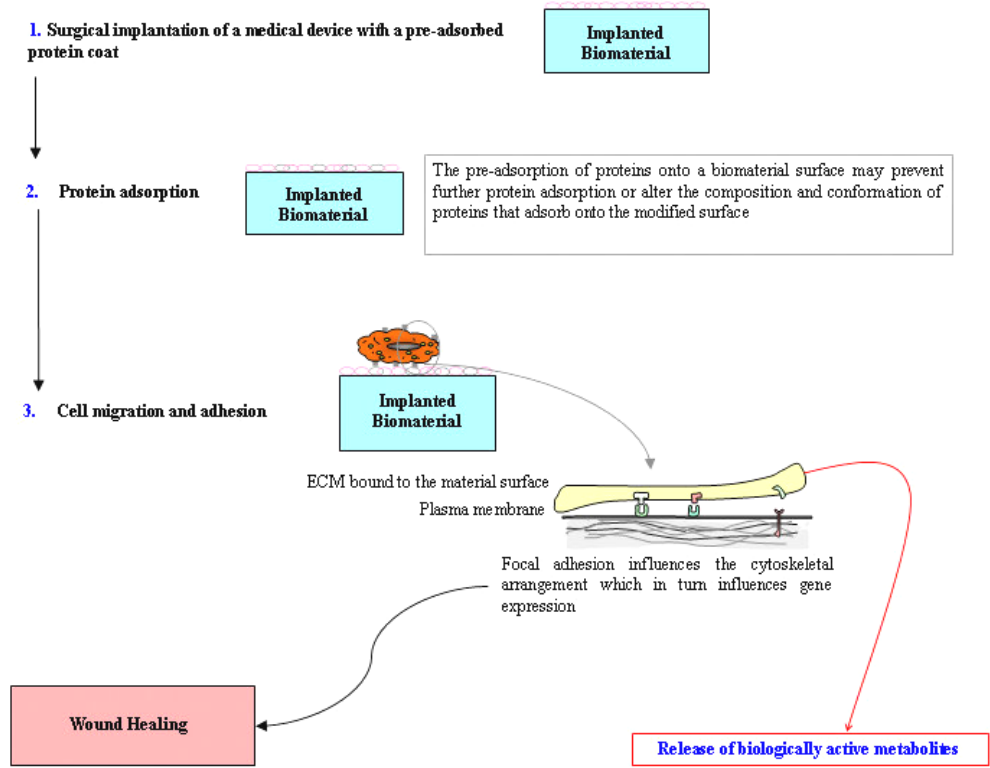


Figure 6. Schematic representation of the events leading to integration following the implantation of a medical device⁶.

Table 9. 10-year patency rates of femoral stems and acetabular cups of total hip prostheses [247]

Mode of Fixation	10-year Patency Rates (%)	
	Femoral stem	Acetabular cup
Cemented	89	93
Apatite-coated	98	90
Porous-coated	92	82
Smooth	68	69

⁶ Cell adhesion to biomaterials is mediated by cytoskeletally associated receptors in the cell membrane which interact with the cell adhesion proteins adsorbed to the material surface from the surrounding biofluids triggering multiple functional biochemical signalling pathways within the cell, e.g. cell-growth, cell shape and cytoskeletal tension, in a manner analogous to cell-cell communication and patterning during embryological development. The potential of this strategy is exemplified by tissue engineering approaches that employ biomaterials with surfaces designed to stimulate highly precise reactions with proteins and cells at the molecular level. Such materials provide the scientific foundation for molecular design of scaffolds that could be seeded with cells *in vitro* for subsequent implantation or specifically attract endogenous functional cells *in vivo*.

Table 10. Comparison of the patency rates of vascular grafts used at various anatomical locations

Anatomical location	Graft	% Patency (Years)
Aortiobifemoral	Dacron [®]	90 (5) ^[119]
	ePTFE	90 (5) ^[119]
Femorofemoral	Dacron [®]	80 (5) ^[119]
	ePTFE	80 (5) ^[119]
Femoropopliteal	Saphenous Vein	70 (5) ^[119]
	Dacron [®]	43 (3) ^[274] 40 (5) ^[119]
	Heparin-bonded Dacron [®]	55 (3) ^[274]
	ePTFE	50 (5) ^[119]
Coronary Artery	Left Internal mammary artery	88 (5) ^[275]
	Saphenous vein	86 (1) ^[276] 74 (5) ^[275]
	ePTFE	59 (1) ^[276]

4. Many diseases of the cardiovascular system require the use of prosthetic materials to replace valves [263] and vascular prostheses [263]. Atherosclerosis is the largest cause of mortality in the U.S. [264] and results in the formation of plaque-like lesions which progressively block the blood vessels as a result of the thickening and hardening of arterial walls. Synthetic blood vessel substitutes such as Dacron^{®*} and ePTFE[♦] grafts are clinically successful when used in high-flow, low-resistance vessels [88, 119, 265] (>10 mm) but show poor patency rates when used to graft small diameter vessels [88, 89, 266, 267] (< 6mm) (Table 10). Small-diameter grafts are prone to early thrombosis because of their lower flow rates and the higher resistance in their outflow vessels with thrombogenicity and stenosis due to intimal hyperplasia being major causes of graft failure [267]. A variety of clinical applications including lower-extremity bypass procedures and coronary artery bypass grafting (CABG require small-diameter grafts (<6 mm)) therefore continue to rely on the use of the autogenous saphenous vein or internal mammary artery (IMA) [119] (Table 10). Bypass grafts are however prone to restenosis proximal to the graft-bypass junction [268] and frequently require secondary surgical procedures *e.g.* stenting and secondary graft procedures which themselves are associated with restenosis, significantly increased mortality rates due to the extended operative times, limited supply of suitable autogenous vessels.
- In the search for an ideal synthetic blood-vessel substitute numerous approaches aimed at improving their long-term patency have been attempted *i.e.*, the modification of the luminal surface of the graft through the use of heparin bonding[219, 269], pre-implantation endothelial cell seeding [6, 270-272] and surface modification to encourage endothelial ingrowth [146, 273]. Advances in haemostasis, thrombosis and vascular biology have also provided a basis for the

* Dacron[®]: polyethyleneterephthalate (PET)
♦ ePTFE : expanded PTFE (polytetrafluoroethylene); Trade names: Teflon[®], Gore-Tex[®] or Impra[®]

development of molecular-designed anticoagulant interfaces and the production of synthetic inhibitors of coagulation and thrombocyte function aimed at improving the haemocompatibility of cardiovascular implants. Additionally the development of hybrid constructs using synthetic materials to form the adventitia and media with a pre-seeded layer of endothelial cells on the inner-luminal surface in contact with the blood show good results with respect to patency [269, 270].

Although these developments in the design of reconstructive biomaterials are extending the longevity of devices, evidence suggests that if dysfunctional tissue can be induced to regenerate or be replaced by newly synthesised tissue that this would offer a significantly superior clinical therapy, would reduce the incidence of secondary complications, negate hypersensitivity issues and hugely impact on paediatric medicine.

Regenerative Medicine

From a biochemical and physiological perspective, tissues metabolise, synthesise and secrete various substances in response to local stimuli. Therefore, the use of non-viable materials to replace tissue results in the loss of biological functionality and/or responsiveness. This loss of biological functionality, the shortfall in donor organ and tissue availability for auto- and allografting and the risk of transgenic transfer from viable xenografts [14] has led to renewed interest in the application of resorbable materials and the development of an entirely new approach to regenerative medicine: tissue engineering. Potential strategies of regenerative medicine include stem cell transplantation, implantation of bioartificial tissues synthesised in the laboratory and the persuasion of the body's own cells to regenerate by rendering the injury environment and/or responding cells regeneration-competent [28]. Tissue regenerative applications include tissues such as skin, cartilage and tendons, ligaments, bone, blood vessels, heart valves, myocardial patches and organs such as heart, pancreas, kidney and liver. To date regenerative therapies for skin and cartilage replacement have been the most successful.

The market for regenerative medicine, although still in its infancy, is rapidly growing and remains an intense area of research that is being driven by the maturation of patents, estimated revenues (*e.g.*, from the cell therapy market alone revenues are expected to exceed 30 billion \$US by 2010 [277]) and clinical demand (*e.g.*, the increasing number of patients worldwide presenting with ulcers (*i.e.*, diabetic ulcers 1,745,000; venous ulcers 2,342,000; pressure ulcers 4,440,000) and hospitalised with burns (1,785,000)). In the past 10 years more than 3.5 billion \$US have been invested worldwide in research and development, mainly by the private sector, in the US [278, 279]. In 2001 annual investment in R&D was 580 million \$US which by 2007 increased to 850 million \$US [280]. The slow uptake of research interest in the tissue engineering field outside of the U.S. pre-2000 resulted in over 70% of the global tissue engineering patents filed between 1980 – 2001 being owned by US-based researchers, followed by 18% in Europe (led by Germany and the UK) and 6% in Japan. This lack of investment has also resulted in the disparity of 55 of the 66 registered tissue engineering companies being US owned (*e.g.*, Advanced Tissue Sciences, Genzyme) compared with 11 European (*e.g.*, Fidia Advanced Biopolymers (Italy), Smith and Nephew (UK), Amara (Germany) BioNova (UK)). To date, however, only a few tissue-engineered devices are

available in the marketplace, primarily in the areas of skin and cartilage regenerative therapies (Table 11) although other therapies are currently at the clinical trials stage [281] (Table 12).

Table 11. An overview of some of the tissue engineering therapies currently applied in regenerative medicine following FDA approval

Year of FDA Approval	Development	Company	Indicated Use
1987	Epicel ^[282, 283]	Genzyme	Only autologous skin graft available; indicated for burn wound closure
1989	Biobrane II ^[284]	Sterling Drug Inc.	Wound dressing
1994	Alloderm ^[285]	LifeCell Corp.	Burn surgery
1996	INTEGRA [®] Dermal Regeneration Template ^[286, 287]	Integra LifeSciences Corp	Acellular dermal regeneration template for burn and reconstructive surgery
1997	TransCyte ^{7 [284, 288]}	Advanced Biohealing	Temporary wound cover for burns
1997	Carticel ^[289-291]	Genzyme	For the repair of clinically significant, symptomatic cartilaginous defects of the femoral condyle caused by acute or repetitive trauma
1998	Apligraf ^[292, 293]	Organogenesis	Non-infected partial and full-thickness skin ulcers; diabetic foot ulcers
2001	OP-1 Implant ^[294, 295]	Stryker	Alternative to autograft for recalcitrant bone non-unions
2001	Laserskin [®]	Fidia Advanced Biopolymers	Biodegradable keratinocyte delivery system
2003	Dermagraft ^{TM [296, 297]}	Smith and Nephew Wound Management	For the treatment of wounds related to dystrophic epidermolysis bullosa ⁸
2006	Oasis Wound Matrix ^[298, 299]	Cook Biotech Inc	Partial and full-thickness wounds, ulcers, surgical wounds
2007	INFUSE ^{TM [300]}	Medtronic	Bone Graft

Table 12. An overview of some of the tissue engineering therapies currently at the clinical trials stage

Company	Product	Application	Reference
Arbios Systems Ltd (formerly Circe Biomedical)	SEPET TM	Liver Assist Device	[301]
	HepatAssist	Bioartificial liver	[302]
VitaGen Inc.	ELAD TM	Bioartificial liver	[302-304]
Alimera Sciences	Iluvien TM	Diabetic macular oedema (DME)	[305]
pSivida Limited	BrachySil TM	Pancreatic cancer	[306]
Tengion	Neo-Bladder Augment TM	Neurogenic bladder	[307]

⁷ Formerly Dermagraft-TC Advanced Tissue Sciences Inc.

⁸ Epidermolysis bullosa is a group of inherited disorders in which skin blisters develop in response to minor injury

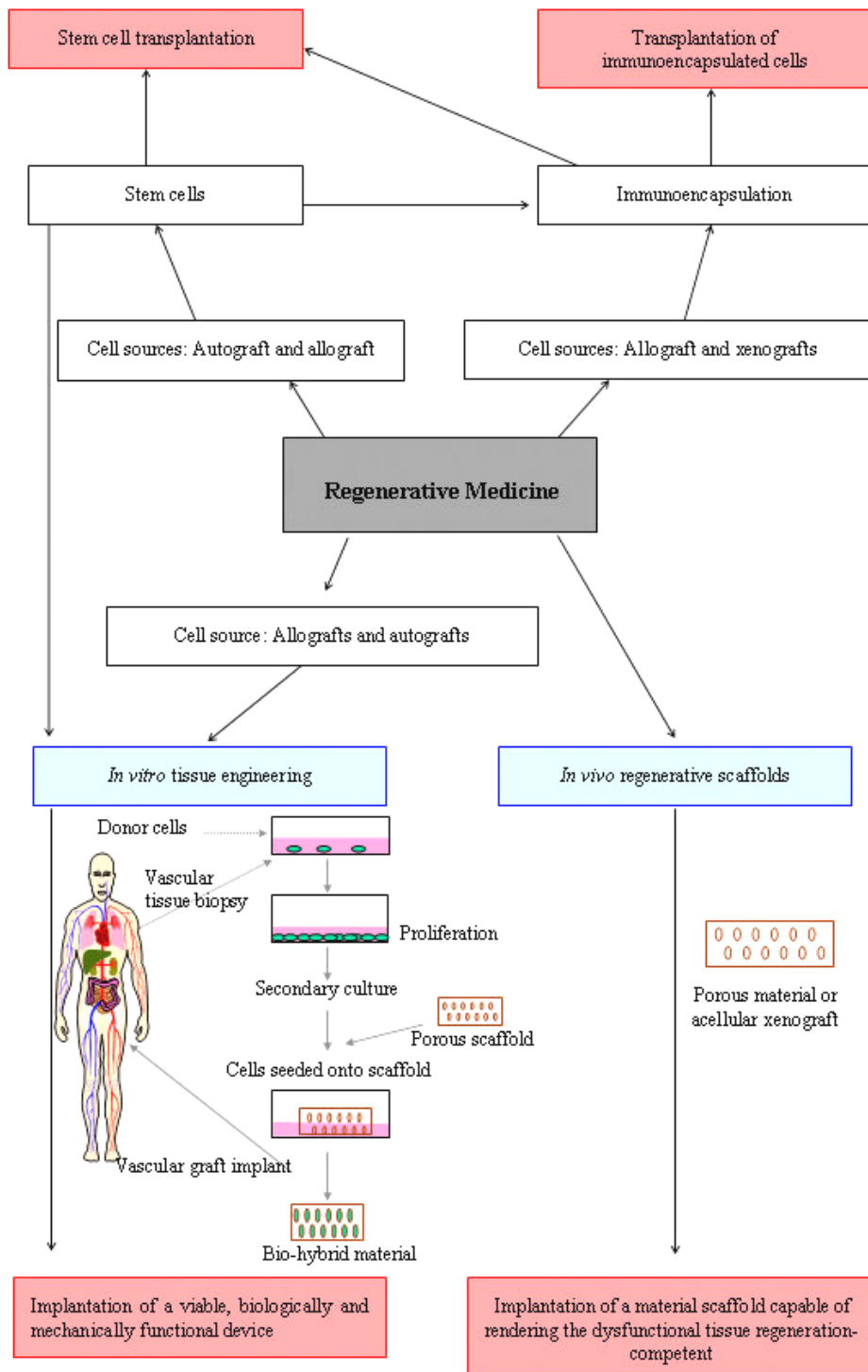


Figure 7. Schematic representation of the interplay of the various fields of regenerative medicine.

Although the term regenerative medicine is often used synonymously with tissue engineering there are application and approach differences that warrant discrimination. This is

further confused by the numerous definitions of tissue engineering cited in literature. Tissue engineering has been defined as:

- *'an interdisciplinary field that applies the principles of engineering and life sciences toward the development of biological substitutes that restore, maintain, or improve tissue function or a whole organ'* [26]
- *'the understanding of the principles of tissue growth, and applying this to produce functional replacement tissue for clinical use'* [308]
- *'the application of the principles and methods of engineering and the life sciences toward the fundamental understanding of structure/function relationships in normal and pathological mammalian tissues and the development of biological substitutes to restore, maintain, or improve function'* [27]
- *'the persuasion of the body to heal itself through the delivery, to the appropriate site, of cells, biomolecules and/or supporting structures'* [309]

If regenerative medicine is considered as being subdivided into: stem cell transplantation, immunoencapsulated cell transplantation, *in vitro* tissue engineering and *in vivo* tissue regeneration (Figure 7) then the underlying principle of the first two approaches is the restoration of biochemical function whilst that of the latter two approaches, which are discussed, is the restoration of architectural, mechanical and biochemical function. Distinction of these approaches in this manner enables a clearer perspective of each areas device design requirements.

In vivo Tissue Regeneration

In this context, various device applications that are designed to induce *in vivo* regeneration by the body's own cells have been identified:

1. Biomaterial barriers to block molecular signals that stimulate scar formation or immune rejection. For example, adhesions (fibrous scar tissue) can form following abdominal and thoracic surgery between the skin and internal organs as a consequence of the normal wound healing response. The presence of a resorbable, non-cellular adhesive polymer film prevents the deposition of a fibrin-rich clot between these layers of tissue preventing the development of adhesions [310-312].
2. Surface modification of a material may be topographic or chemical. Topographic modification of surfaces includes the modification of the surface texture [313] or roughness [314, 315] which influences cell adhesion, proliferation, orientation and biochemical activity. Chemical modification of surfaces includes the immobilisation of bioactive ligands that may be micro-patterned to modulate cell behaviour [316-320] (biomimetic materials):
 - receptor-mediated control of single and multiple cellular morphologies and functions *e.g.*, by RGD [321, 322] and YIGSR [323] sequences [273]
 - controlled growth factor/cytokine release from devices or transplanted cells to control tissue regeneration:
coating or incorporation of bone morphogenic protein on or into bone void fillers [324-327]

- covalent immobilisation of growth factors *e.g.*, epidermal growth factor (EGF) [328, 329] and VEGF [330, 331]
- a combination of growth factors such as VEGF and BMP-2 improves bone formation and bone healing [332]
3. Scaffolds or matrices to control and guide wound healing and tissue regeneration. Scaffold material options are diverse and may include:
- Autograft, allograft, demineralised bone matrix
 - Acellular xenografts: *e.g.*, SIS [16-18, 21]
 - Biopolymers: *e.g.*, collagen [333], fibrin, hyaluronic acid, alginate [334, 335], chitin/chitosan [336-339]
 - Ceramics: *e.g.*, Coral, hydroxyapatite [340], Tricalcium phosphate
 - Synthetic polymers: Poly(lactide) PLA [341], Poly(lactide-co-glycolide) (PLG) [342], caprolactones, methylcellulose, polyesterurethanes [146]
 - Glasses and glass ceramics *e.g.*, resorbable Bioglass® [121, 343]
 - Composites: *e.g.*, Poly(lactide-co-glycolide)/hydroxyapatite [344], collagen-hydroxyapatite [345], collagen-calcium phosphate [346], polyurethane /poly(lactic-co-glycolic) acid [347]
 - Smart matrices: scaffolds combined with immobilised bioactive ligands that induce tissue ingrowth *in vivo* as indicated in c. above.

The following discussion is primarily limited to the use of scaffolds fabricated from ECM polymers, *i.e.* collagen, and its derivative gelatin, hyaluronic acid, fibrin, elastin and composites of these materials, for *in vivo* tissue regenerative applications. The primary advantages of this approach are the reduced number of operations required (*i.e.* no donor tissue is required to be harvested) and therefore reduced recovery time for the patient, there are no issues with regard to cell sourcing, and the degradation products of naturally-derived ECM polymer are readily cleared by the host and are non-immunogenic.

In vivo tissue regeneration relies on the implantation of a biomaterial scaffold into which local or circulating regeneration-competent cells migrate and proliferate. The success of such an approach relies on the inter-relationship between the cell type(s) required to colonise the scaffold, the scaffold-tissue interface and the scaffold.

Table 13. Regenerative capacity of cells following trauma [348]

Category	Normal rate of replication	Responses to stimulus/injury	Examples
Renewing	High	Modest increase	Epithelium Intestinal mucosa Bone marrow
Expanding	Low	Marked increase	Endothelium Glandular epithelial Vascular smooth muscle Osteoblasts Fibroblasts Liver cells
Static	None/Rare	No replication; replacement by scar	Heart muscle cells Nerves of the CNS

The regenerative capacity of tissue cell populations is varied and classified, as indicated in Table 13, as continuously regenerating (renewing) (*e.g.*, the lining cells of the

gastrointestinal tract (every 3 days) or the dermis (every 14 days) [349]), inducibly regenerative (expand rapidly in response to tissue trauma *e.g.*, osteoblasts) or static (these cells appear to be non-regenerative *i.e.*, they have become terminally differentiated). The success of *in vivo* tissue regenerative strategies may be predicated by the presence of cell populations with inducible regenerative capacity within the tissue of interest *i.e.*, adult stem cell and progenitor cell populations (*e.g.*, epithelium of the respiratory and digestive tracts, liver, skin keratinocytes) or tissues that can regenerate by compensatory hyperplasia (such as the liver and the β -cells of pancreatic islets) [28]. However, recent evidence suggests that some regeneration of reputedly static cell populations, *i.e.*, neural [350, 351] and heart [352], can occur under specific conditions and a degree of nerve regeneration [353], although imperfect, has been achieved using an *in vivo* tissue regenerative template [354]. Therefore, the potential clinical applicability of this approach is still speculative.

The scaffold for *in vivo* tissue regeneration must in general fulfil the following criteria [355, 356]:

1. Biocompatible, resorbable materials that resorb in a controlled manner
2. Structure should be open with high porosity and capable of inducing rapid angiogenesis and cellular invasion
3. Manufacturing must be easy, reliable and reproducible
4. Possess appropriate functional properties including appropriate mechanical properties, adherence to the surrounding host tissue, provide a mechanically stable architecture on which cells can proliferate and transfer appropriate physiological mechanical stimuli to the invading cells.

a. Biocompatible, resorbable materials for *in vivo* tissue regenerative scaffolds

The natural scaffold in the body is the extracellular matrix (ECM) the composition and structure of which varies from one tissue to the next *e.g.*, the basal lamina (basement membrane) directly underlying epithelial cells contains laminin, collagen, fibronectin, vitronectin whilst stromal tissue (interstitial matrix) contains matrix-secreting cells (fibroblasts, osteoblasts), collagen, elastin, fibrillin, fibronectin, vitronectin, GAGs⁹, glycoproteins and regulatory proteins. Typically these matrices are highly hydrated macromolecular networks that may be envisaged as fibre-reinforced composites composed of various amounts of fibrillar proteins (*e.g.*, collagen (Types I, II and III) and elastin), glycosaminoglycans (*e.g.*, hyaluronic acid, chondroitin-4-sulphate, chondroitin-6-sulphate, dermatan sulphate, heparin and heparin sulphate) and adhesion proteins (*e.g.*, fibronectin and laminin) [348]. The physico-mechanical properties of the ECMs are largely determined by these matrix-forming polymers as they control the tissue integrity, physiology and mechanical properties, *e.g.*, collagen is primarily responsible for the tensile strength of tissue [348], but the ECM polymers also play a crucial role in cell behaviour. The ECM influences cell adhesion, proliferation, migration, differentiation and apoptosis via an array of transmembranal cell surface receptors including integrins [357] (such as the $\alpha 5 \beta 1$ integrin that binds to RGD sequences that are found on several ECM molecules including fibronectin), proteoglycans receptors (*e.g.*, CD36 and CD44) and non-integrin laminin receptors [323] (that bind to sequences such as YIGSR in laminin) that transmit signals intracellularly via the

cytoskeleton modulating gene expression [358]. For example, integrins on the surface of cells are low affinity receptors that are present in high copy numbers so that, in general, they can bind weakly to a range of different but related matrix molecules promoting cell-cell interactions and cell-ECM matrix binding [348, 359]. Cell proliferation and differentiation are also modulated by various soluble growth factors and interleukins [360]. Scaffolds for *in vivo* tissue regeneration are not only required to replicate the multifaceted physicommechanical functions of the ECM but are also required to modulate the cell-material interfacial response in a manner analogous to bioactive materials such that specific cell phenotypes adhere to and proliferate on the scaffold synthesising *de novo* tissue-specific ECM which then acts as the tissue regenerative template.

As materials intended to be used as matrices in tissue engineering have to imitate the properties of the tissues that they are replacing it is reasonable to explore the use of these polymers in the development and design of ECM analogues. Physically or chemically modified naturally-derived hydrogel-forming materials, *e.g.*, hyaluronic acid, collagen and gelatin, have frequently been used in tissue engineering applications (Table 14) because they are either components of, or have macromolecular properties similar to, the natural ECM. Three key characteristics of the degradation and resorption process of these materials influence performance:

- (i) The rate at which the scaffold loses its mechanical properties
- (ii) The rate at which the scaffold is removed from the implantation site
- (iii) The nature and concentration of the soluble products that are released into the site as the material is broken down.

The controlled degradation of the mechanical properties alone is a major challenge but in general, most design strategies tend to extend the degradation time over months in order to minimise the risk of early failure [349].

b. Scaffold morphology

One of the primary functional roles of an *in vivo* tissue regenerative scaffold is the definition of the area on or in which new tissue can be laid down by providing structural support and voids. The bulk morphology of the scaffold guides the structure of newly synthesised tissue by controlling its size, shape and vascularisation [361] while the microporosity of the scaffold (pore size, pore shape and volume fraction) affects the rate of the fibrovascular ingrowth [362, 363], a key determining factor in governing the inflammatory response [364].

Microporosity has long been known to influence cell behaviour at the material-tissue interface *e.g.* high porosity in large diameter vascular prostheses such as ePTFE (IND > 45 µm) encourages neointima formation promoting clinical performance [407, 408]. Cellular adhesion and growth on scaffolds and phenotypic expression have also been found to be influenced by pore size and distribution in a cell-type dependent manner [409] (Table 15). Heterogeneity in the pore size and distribution leads to patchy cell adhesion which results in the production of a biomechanically inferior ECM compared with cell growth and ECM production on scaffolds with a uniform pore structure [410]. Additionally, while the shape of

⁹ GAGs = glycosaminoglycans

the pores affects cell coverage of the scaffold surface *i.e.*, cells aggregate into spherical structures on scaffolds with equiaxed pores while on scaffolds with elongated pores the cells align with the pore axis resulting in reduced biosynthetic activity. Scaffold chemistry and compliance also influence cell behaviour *e.g.*, the *in vitro* seeding of chondrocytes on Type I and Type II collagen scaffolds of equivalent bulk porosity and pore sizes results in differing cell morphologies and biosynthetic activity [386] while the *in vitro* seeding of human mesenchymal stem cells (hMSC) on a collagen-coated polyacrylamide gel with varied degrees of crosslinking, and hence elasticity, results in the hMSCs being induced to differentiate into different tissue lineages [411].

Table 14. Potential applications of biopolymers in tissue engineering applications

Biomaterial	Format	Application	Reference
Hyaluronic acid	Gel	Nerve Regeneration	[365]
	Microspheres	Drug/growth factor delivery	[366, 367]
	Film	Controlled peptide release and protein delivery	[368]
		Adhesion Prevention	[369-375]
	Sponge	Cartilage	[368, 376, 377]
		Wound Healing	[199]
Collagen	Microsphere	Drug/growth factor delivery	[378, 379]
	Film	Heart valves	[337, 380, 381]
	Sponge	Cartilage	[382-384]
		Wound Healing	[385-390]
		Nerve regeneration	[391]
		Bone regeneration	[392, 393]
		Urethral repair	[394]
		Small calibre vascular graft	[395]
Gelatin	Gel	Wound Dressing	[396]
	Microsphere	Drug/growth factor delivery	[397-400]
	Sponge	Articular Cartilage	[401]
		Nerve Regeneration	[402]
Collagen-glycosaminoglycan	Sponge	Nerve Regeneration	[403, 404]
		Tendon Regeneration	[405]
		Skin	[406]

Table 15. Optimum pore size for cell specific ingrowth into porous matrices

Cell Type	Pore Diameter (µm)	Reference
Fibroblast	5 - 20	[412-414]
Hepatocytes	20	[412]
Adult mammalian skin	20 - 125	[406]
Endothelial	60 - 80	[407]
Bone Matrix regeneration	80 - 250	[349, 410]

The scaffold provides the initial surface and mechanical support onto which cells can grow and provide pathways for mass transport. In all settings the mass transport of metabolic substrates (oxygen, glucose and amino acids) into the bulk material and the clearance of products of cell metabolism (carbon dioxide, lactate and urea) are critical for cell survival. This flux of metabolites in and out of most metabolically active tissues is primarily influenced by passive diffusion along concentration gradients. Because the diffusion of oxygen is relatively slow and its consumption high its local tissue concentration becomes the primary limiting factor in cell survival. In metabolically active tissue, the local oxygen concentration is approximately 0.07 mM and oxygen diffusion distances between a capillary lumen and a cell membrane is 40 – 200 μm [349]. Therefore, the thickness of a non-vascularised graft to support cell viability in its central region will be limited by the local oxygen tension. Mathematical modelling of this suggests that a 1 cm thick graft will support 4 times as many viable cells as a 2 cm thick graft but that this number is still 100 – 1000 fold less than the number of cells found in bone or bone marrow aspirates [349]. Potential strategies for overcoming this diffusion barrier are the incorporation of nanostructural features to aid mass transport and the promotion of angiogenesis by the release of angiogenic factors from the scaffold in a spacio-temporally controlled manner at a rate commensurate with progenitor cell infiltration so that the new vasculature can support the mass transport requirements of the invading connective cells [415].

c. Approaches to scaffold manufacture

A major goal in fabricating scaffolds for tissue regeneration is the accurate control of pore size and porosity (>90%) within optimal limits. Various fabrication routes have been applied to address these design requirements including salt leaching [416], solvent evaporation by freeze drying [417-420], solvent-casting and particulate leaching [421, 422], solvent-casting and critical point drying [423], supercritical fluids [424], woven/non-woven fibres [199], membrane lamination [425], fibre bonding [416, 426], phase inversion processes such as liquid-liquid phase separation and liquid-solid phase separation [427], electrospinning [428, 429], *in situ* polymerisation [430], melt molding [431], sintering of compacted powders, 3-D printing techniques [432, 433], fused deposition modelling and sublimation [434]. Particulate leaching, freeze drying, gas infusion and phase separation fabrication methods lead to the creation of isotropically distributed voids and connected pores while solid free-form fabrication methods including 3-D printing processes and stereolithography create strategically orientated channels and pores with defined macroscopic shapes.

A combinatorial approach of phase separation and freeze-drying is frequently applied in the production of porous hydrogels. In the case of hydrogels, where water is the solvent, ice crystals formed during the freezing process are removed by sublimation during freeze-drying producing pores. The size and number of ice crystals formed during the freezing process govern the diameter, shape and distribution of pores formed in the scaffold [390]. The number, size, homogeneity and rate of growth of ice crystals, and consequently the 3-D porous network, is in turn influenced by the polymer volume fraction, sample volume, rate of freezing and solvent [419]. As each of these 'recipes' possesses a unique heat-transfer rate the scaffolds produced will display diverse morphological and bulk properties.

d. Scaffold functional requirements

Initially a scaffold needs to function in a manner analogous to the tissue that it is replacing and therefore it must possess the appropriate mechanical properties as with conventional replacement materials. This results in two conflicting design parameters in that the strength of a bulk material is reduced by the presence of voids whilst a scaffold's porosity is necessarily high for cell infiltration. Additionally, as cells infiltrate the scaffold they must also be exposed to a combination of biochemical and biomechanical cues reminiscent of that found during embryonic development for tissue-like architectures to be formed. Biomechanical interactions, or mechanotransduction, between the ECM and cells stimulation of cells influences tissue formation, cell adhesion, shape, intracellular biochemistry and gene expression [435-439] which, when in conjunction with exogenous growth factors such as TGF- β , have been shown to stimulate increased ECM synthesis by smooth muscle cells in Type I collagen gels *in vitro* [435]. Stress-induced cell behavioural changes, including cytoskeletal traction, have also been induced by scaffold microtopography [440], which results in enhanced responsiveness to surface-tethered adhesive proteins such as fibronectin.

In vitro Tissue Regeneration

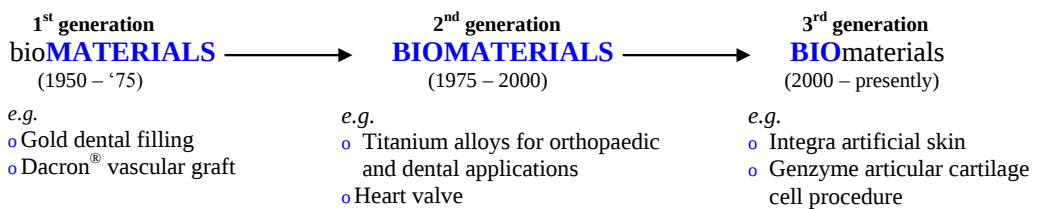
Recent advances in the development of small-diameter artificial arteries are discussed here to illustrate the impact of *in vitro* biomechanical and exogenous biochemical conditioning on the mechanical properties of *in vitro* tissue engineering scaffolds [441-443] (Table 16). Other studies have also shown the impact of biomechanical conditioning on cell proliferation, biosynthetic activity and phenotype in cartilaginous and musculoskeletal tissue and on cardiomyocytes and cardiac fibroblasts [444].

The autologous saphenous vein or left internal mammary artery are generally harvested for coronary artery bypass surgery with clinical patency rates of 74 and 88 % at 5 years, respectively. Differences in the patency rates have been attributed to compliance mismatch of the vessels which leads to turbulent flow at the anastomoses and anastomotic intimal hyperplasia. However, 10 – 40% of patients do not have a suitable vessel for harvesting due to size mismatch, previous procedures or venous disease and, as the patency rates of current synthetic grafts are poor in small calibre blood vessels, there is a need for an alternative. The mechanical requirements of completely biological tissue-engineered grafts are required to not only demonstrate physiological burst pressure and compliance but they must also be resistant to rapid degradation and fatigue-induced aneurysm formation *in vivo* [445] if graft longevity is to be achieved. The impact of fabrication on both the mechanical properties and *in vivo* performance of tissue engineered small diameter vascular grafts are presented in Table 16 and their mechanical properties compared with those of native tissues. Comparison of the burst strength of native versus decellularised porcine carotid arteries indicates the impact cells, primarily the smooth muscle cells, have on the mechanical properties of the tissue. *In vitro* the stimulation of cell growth under pulsatile conditions is seen to result in a 6.5 fold increase in the burst pressure of PGA-PHA grafts [441], while in a further study [446] the wall thickness of a PGA-based graft was reported to be significantly lower in static versus pulsatile cultures, 230 versus 380 μm respectively. This latter study also examined the impact of supplementation of the media with exogenous factors and reported a 7-fold increase in the burst strength of grafts grown in supplemented media. In a more recent study [445] a graft grown under pulsatile conditions *in vitro*, entirely through the manipulation of the cell

biology of autologous cells, has resulted in the production of grafts whose burst pressures are comparable to that of the porcine carotid artery with a compliance intermediary of that of the saphenous vein and internal mammary artery. This latter approach is a significant breakthrough in *in vitro* tissue engineering but the graft's lengthy production time and how remodelling of the implanted graft will influence its rate of biodegradation and resistance to fatigue will ultimately determine its clinical applicability.

CHALLENGES AND OPPORTUNITIES

The metamorphosis of the biomaterials' field over the last 50 years to meet the changing and increasingly sophisticated clinical demands was succinctly conveyed by Anderson [452] when he described the development of applied biomaterials as having gone through the transitional changes of:



This perspective clearly identifies the change in the primary character of applied biomaterials.

The speed of change in the biomaterials' field over the past 10 years has primarily been driven by advances in molecular and cell biology with the result that many 1st generation biomaterials have been modified and new materials developed which possess improved interfacial interaction with the host tissue for many traditional applications. Future research and development of biomaterials for surgical replacement is likely to focus on the molecular, cellular and tissue interactions with materials and minimally invasive surgical approaches.

In regenerative medicine, biomaterials continue to play a pivotal role in the development of new clinical therapies. With the continued changes in awareness of the complexities faced in attempting to regenerate human tissue, tissue-engineering may be considered as being 'the application of viable or non-viable bioactive biomaterials to correct degenerative or pathological conditions so that the native tissue functionality and architecture is restored'. One of the major obstacles to overcome in achieving this goal is the spacio-temporal control of tissue-specific cell phenotypes. It is however hoped that, by applying engineering design principles in conjunction with advances in cell and molecular biology, approaches towards reconstructive or regenerative repair will be found for many tissue defects but these technical advances must be considered in the context of the risk/benefit and cost for the patient. Furthermore, with increased understanding of the cell biology of pathological states it is conceivable that in some instances surgical repair may be obviated by pharmacological intervention *e.g.*, the use of anti-VEGF in the treatment of PDR [58]

Table 16. Summary of recent advances in vascular engineering (adapted from reference 447)

Tissue/Scaffold Type	Wall thickness (µm)	Scaffold Material ¹	Cells ²	Cell Growth conditions	Burst Strength (mm Hg)	Compliance (%)	Implantation site	Outcome	Ref
Saphenous Vein	250	Native tissue			1680 ± 307	0.7 – 1.5			[445]
Human Artery	350 - 710	Native tissue			2031 - 4225	4.5 – 6.2			
Porcine carotid artery (Proximal)	810	Native tissue			3124 ± 158				[448]
	519	Decellularised native tissue			2338 ± 245				
No Scaffold			HUVEC, HUVSMC and HSF		2594 ± 501		Canine femoral artery	50% at 7 days	[449]
	407 ± 49	None	Human fibroblasts and EC	Pulsatile	3468 ± 500 ^a	1.5 ± 0.3 ^a	Abdominal interpositional graft in rats	85% at 225 days	[445]
Synthetic Scaffold		PGA	Bovine aortic SMC and EC	Pulsatile – supplanted media	< 300 ^b		Swine saphenous artery	Non-pulsed: thrombosis	[446]
	380			Pulsatile + supplemented media	2150 ± 705 ^b			Pulsed patent at 4 weeks	
		PGA-CL/LA	Canine femoral vein SMC and fibroblasts				Canine inferior vena cava	100% at 13 months	[450]
		PGA-PHA	Ovine carotid SMC, EC and fibroblasts				Ovine infrarenal aorta	100% at 5 months	[198]
		PGA-PHA	Ovine carotid EC and myofibroblasts	Static	50 ± 5 ^c				[441]
				Pulsatile	326 ± 5 ^c				
Biologically-derived Scaffold	750	Uncrosslinked collagen	HDFs	Static	90 ± 10 ^d				[451]
	750	GTA Crosslinked collagen			650 ± 170 ^d				

¹ PGA = polyglycolic acid, CL = caprolactone, LA = lactic acid, PHA = poly-4-hydroxybutyrate

² HUV = Human umbilical vein; EC = endothelial cells; SMC = smooth muscle cells; HSF = human skin fibroblasts, HDFs = human dermal fibroblasts

a = 28 weeks; b = 8 weeks; c = 4 weeks; d = 8 days

Finally, in the research and development of new and improved materials there is also the ongoing issue/dilemma regarding predictive *in vitro* evaluation of clinical performance. This is illustrated by the recent termination of clinical trials with polyurethane small diameter grafts due to increased biodegradation *in vivo*. As current biological test results are test-method and biological model selection-specific, results of such tests may be instructive but may lack correlative power to the clinical safety and efficacy of an implant. Therefore, with the development of biomaterials with increased interfacial tissue interaction and combinatorial biologic-biomaterial tissue engineered implants comes the need for the development of new perspectives and approaches towards the biocompatibility or safety assessment. For example, the behaviour of cells in 2-D cultures is significantly different from that in 3-D cultures (which more closely reflect tissue architecture); the clearance of injected hyaluronan in an arthritic joint occurs at a significantly increased rate due to the increased activity of neutrophils in this inflammatory disease. Equally, without clinical trials, the 'true' long-term physiological performance of biomaterials cannot be monitored and therefore the take-up of new approaches to repair may have a minimum of a 10 year lead-time post clinical trials. Nonetheless, despite the numerous technical difficulties that remain to be solved the potential for future developments in this field remain both challenging and exciting.

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Chapter 5

BIOMATERIALS IN BLOOD-CONTACTING DEVICES: COMPLICATIONS AND SOLUTIONS

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ABSTRACT

All blood-contacting medical devices in use today are subjected to some degree poorer blood compatibility than the native artery. Hemostatic mechanism, arresting bleeding from injured blood vessels, induces platelet adhesion and activation onto artificial biomaterials, which leads to undesirable outcomes such as blood clotting at the site of the implant, continual shedding of thrombi, and depletion of platelets from the blood stream. Such complications have hampered the clinical success of blood contacting devices, limiting the patency of small-diameter vascular grafts and making necessary the use of anticoagulants in patients undergoing extracorporeal bypass or synthetic heart valve implantation. Therefore, development of non-thrombogenic biomaterials is in great need for blood contacting devices. The current approaches mainly focus on surface modifications with biological anticoagulants such as heparin, or anti-fouling molecules like poly(ethylene oxide). In this review, we will first introduce the blood components involved in hemostasis and thrombosis, followed by the common biomaterials applied in blood-contacting devices. Next, the complications induced by the interactions between blood and biomaterials will be briefly addressed. Finally, the commonly used techniques for improving biomaterials' hemocompatibility will be expatiated.

1. INTRODUCTION

The technological advances in recent years were witnessed by the wide variety of new and exciting biomaterials currently used in medicine and biotechnology. For devices in contact with blood, the ideal characteristics can be discussed in terms of mechanical properties, chemical properties and hemocompatibilities [1, 2]. For implantable grafts, the

satisfactory mechanical properties include the structural integrity, compliance and durability, as well as the resistance to body environment. The preferential chemical characteristics comprise inertness, minimum reactivity and no release of toxic substance that may destroy the components in blood. Moreover, hemocompatibility related to the specific interactions between biomaterials and circulating blood is required for blood-contacting devices. The requirements for hemocompatibility include stability in blood flow, no induction to the activation of blood components, and no provocation of humoral and cellular immune-response and inflammatory responses [3, 4].

Currently, the most frequently utilized blood-contacting medical devices are usually divided into categories as catheters, blood vessel grafts, vascular stents, artificial heart valves, circulatory support devices, various extracorporeal tubings, hemodialysis, hemapheresis, and oxygenator membranes [2, 5]. Although much efforts have been paid to improve the blood compatibility for biomaterials, all blood-contacting devices in use today are still subjected to some degree incompatibility compared to the native organs. Therefore, in practice, the patients implanted with long-term blood-contacting devices are subjected to heparin and hirudin, which might cause life-threatening events such as hemorrhage, local irritation and osteoporosis [6, 7].

The biomaterial scientists have focused on improving the hemocompatibility according to the following two strategies. The first one is to create non-fouling surfaces that repel biomolecules (proteins, platelets and cells) therefore prevent or suppress uncontrolled reactions such as activation of the blood coagulation cascade, and aggregation of adherent blood platelets. The second strategy is to prepare surfaces to be inert or passive with respect to blood reactions.

This review aimed, first of all, to detail the coagulation process at the molecular level for all components in blood. The coagulation mechanisms and the relationships between biomolecules in blood during the formation of thrombus are elucidated. Moreover, because the executions and administrations for anti-thrombogenic depend highly on the blood-contacting duration, the targeted organ or tissue, the synthetic polymers utilized frequently for medical devices are discussed in terms of their chemical structures, physical-chemical characteristics and applications. Finally, after the detailed summary of the interactions between blood and biomaterials, comprehensive illustrations on the common methods conducted to improve blood compatibility of biomaterials are demonstrated.

2. BLOOD COMPONENTS INVOLVED IN HEMOSTASIS AND THROMBOSIS

Blood is composed mainly of a variety of blood cells such as red blood cells, white blood cells and platelets, salts, and many proteins including albumin, immunoglobulin, and various clotting factors. Upon injury, the exposed subendothelium initiates the innate hemostatic mechanism to arrest bleeding from the damaged blood vessel. Hemostasis involves a complex set of inter-dependent reactions between (1) the sub-endothelial surface of an injured blood vessel, (2) circulating platelets, and (3) clotting factors. During coagulation, the clotting factors are activated, and finally result in the formation of an enzyme called thrombin. Thrombin converts the soluble protein fibrinogen to insoluble fibrin, which acts as the

foundation of mature thrombus by providing a scaffold for the entrapment of platelets, blood cells, and plasma proteins. The formed thrombus may be subsequently removed by fibrinolysis. In this section, the blood components involved in hemostasis and thrombosis will be briefly introduced.

2.1. Platelets

Platelets play a critical role in recognition of vascular injury, formation of effective hemostatic plugs, retraction of clots and wound healing. The discoid cells, roughly $1 \times 3 \mu\text{m}$, circulate in the blood with a life span of 8-10 days with concentration of $2.5 \pm 0.8 \times 10^8/\text{ml}$ in human blood. General reviews describing the platelet can be found in the literature and textbooks [8, 9]. Platelets contain at least three types of secretory granules: α -granules, dense granules, and lysosomal granules. The α -granule, the most abundant type, is a unique secretory organelle, containing many adhesive proteins (e.g. fibrinogen, fibronectin, von Willebrand factor, and thrombospondin), plasma proteins (e.g. immunoglobulin and albumin), cellular mitogens (e.g. platelet derived growth factor and TGF- β), and clotting factors (e.g. factor V) [10]. Furthermore, the membrane of the α -granules contains a number of receptors including glycoprotein GP IIb/IIIa ($\alpha_{\text{IIb}}\beta_3$) and P-selectin. The dense granules contain ADP, serotonin, Ca^{2+} , and phosphates, which are released when platelets receive strong stimulation. Lysosomal granules contain numerous degradative enzymes, and may play a role in clot dissolution.

Since the primary function of platelets depend on their adhesive interactions, platelet activities are mediated by many membrane glycoproteins that are receptors for adhesive proteins. For example, GPIb/IX mediates platelet adhesion to the subendothelial matrix-bound von Willebrand factor (vWF). The most abundant glycoproteins on the platelet membrane is the integrin family, including GP IIb/IIIa, GP Ia/IIb and $\alpha_v\beta_3$ [11]. Platelet aggregation is mediated by GP IIb/IIIa, a cation-dependent heterodimeric receptor for fibrinogen, fibronectin, vitronectin, vWF and thrombospondin [12]. GP IIb is composed of a larger subunit (GP IIb $_{\alpha}$, 125 kDa) and a small transmembrane disulfide-linked subunit (GP IIb $_{\beta}$, 25 kDa) [13]. GP IIIa is a single polypeptide chain with a molecular weight of 95 kDa. The number of GPIIb/IIIa receptors is approximately 80,000 per platelet and about 70% of the GPIIb/IIIa complex is present on the platelet membrane. In addition, platelets express four other integrins on their surfaces including $\alpha_v\beta_3$, which binds similar ligands to GP IIa/IIIa, and three members of the β_1 integrin family: GP Ia/IIa, GP Ic/IIa ($\alpha_5\beta_1$), and GP Ic'/IIa ($\alpha_6\beta_1$), which bind collagen, fibronectin, and laminin, respectively [11].

Platelets normally stay in the quiescent state in blood circulation until stimulated by attaching to the exposed sub-endothelium or by soluble platelet agonists. Platelet activation is recognized by their stickiness, changes in cell shapes, contraction and release of granule contents, and irreversible aggregation. Upon activation, the morphology of an unactivated platelet, discoid shape maintained by a circumferential bundle of microtubules, is transformed to a more spherical form, sometimes with a few pseudopodia, due to disassembly of the microtubules. The activated platelet becomes sticky to one another, extends pseudopodia and lamellapodia, and eventually spreads itself in an irreversible process. Eventually, the platelet reaches a fully spread morphology [14, 15] (Figure 1).

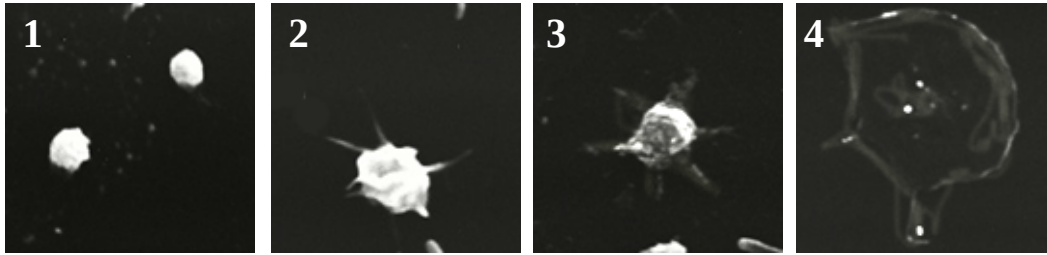


Figure 1. The SEM images of activated platelets. After activation, the morphology of a platelet is transformed to round (1), dendritic (2), partial spreading (3) and fully spreading (4).

Activated platelets release the contents of their α granules, ADP and serotonin from the dense granules, and initiate the formation of a small amount of thrombin and thromboxane A_2 . All of the above molecules facilitate recruitment of platelets for the formation of platelet aggregates. The stimulation of circulating platelets by these agonists causes the expression of activated GP IIb/IIIa, binding fibrinogen to support platelet aggregation in a Ca^{2+} -dependent manner. Platelets will not aggregate in the absence of soluble plasma fibrinogen, membrane receptor GP IIb/IIIa, or Ca^{2+} . Aggregated platelets possess procoagulant activity and facilitate coagulation, which will be discussed later.

2.2. Fibrinogen and von Willebrand Factor

Fibrinogen (FGN) is a dimeric molecule consisting of three disulfide-linked polypeptide chains, designated as $A\alpha$, $B\beta$, and γ . Each set of three polypeptide chains is composed of 1482 amino acid residues, and the computed molecular weight of FGN is 340 kDa. The concentration of FGN in human plasma is approximate 2.5~3.0 mg/ml. Two main hemostatic functions of FGN include fibrin formation and platelet aggregation. The mechanism for fibrin formation will be introduced in the succeeding section.

Each FGN molecule possesses three pairs of putative platelet binding sequences, two Arg-Gly-Glu (RGD) sequences in the $A\alpha$ chain (RGDF at $A\alpha$ 95-98, RGDS at $A\alpha$ 572-575) and a dodecapeptide at the carboxy-terminus of the γ chain (HHLGGAKQAGDV at γ 400-411) [16, 17]. RGD peptide is a common cell recognition sequence in many cell adhesive proteins, while the dodecapeptide is only recognized by platelets, but not by other cell types such as endothelial cells [18]. The three putative binding sites for platelets in FGN have been deduced from indirect evidence, in which synthetic peptides containing the RGD sequence or the dodecapeptide were shown to inhibit platelet aggregation and attachment to adhesive ligands such as FGN and vWF [19-21]. Both RGD sequences and HHLGGAKQAGDV bind to GP IIb/IIIa, but interact with different domains of the integrin [22-24], where the binding of the two RGD peptides and the dodecapeptide is mutually exclusive. On the basis of mutagenesis experiments, it has been proposed that the two RGD sequences are neither necessary nor sufficient to support platelet aggregation, and only the carboxyl terminal region of the γ -chain has been identified as an essential binding site for this activity of FGN [25, 26]. It has been speculated that the three platelet-binding domains of FGN molecule cooperatively enhance the affinity of FGN for platelets. Furthermore, FGN can serve as a bridge between

platelets due to its multiple binding sites for GP IIb/IIIa [16]. The binding of FGN to flowing platelets depends on platelet activation. GP IIb/IIIa becomes effective in binding soluble FGN or the other ligands only after platelets are activated by platelet agonists such as thrombin or collagen.

von Willebrand factor (vWF) is a glycoprotein that circulates in human plasma in the form of multimers ranging from 500 to 20,000 kDa. The mature vWF subunit contains 2050 amino acid residues and as many as 22 carbohydrate side chains. vWF is present in human plasma at a concentration approximately 200-fold lower than that of FGN. Three primary interactions of vWF are known to have functional significance in hemostasis. vWF interacts with Factor VIII, extracellular matrix and platelets. It is believed that Factor VIII is stabilized in circulation by association with vWF. Factor VIII must be dissociated from vWF to express its cofactor activity in the coagulation cascade [27].

vWF plays an important role in mediating platelet interaction with the damaged vessel wall under high shear rates [28]. When the endothelial linings of vessel walls are damaged, the underlying subendothelial structures are exposed to the blood elements, and vWF binds to collagen and other extracellular matrix components. The bound vWF is believed to undergo a conformational change, allowing vWF to bind unactivated platelets via platelet membrane integrin GP Ib [29]. Two regions of the vWF subunit comprising residues 474-488 and 694-708 are involved in the binding of GP Ib [30]. The interaction between vWF and GP Ib leads to platelet activation and calcium release inside the platelet, which subsequently results in activation of GPIIb/IIIa. vWF further binds to activated GP IIb/IIIa via the RGD sequence in its carboxyl-terminal region (1744 – 1746), which leads to platelet spreading and irreversible platelet adhesion. The binding of vWF to GP IIb/IIIa also mediates platelet aggregation. Santoro showed that the binding of surface-bound vWF to GP Ib and GP IIb/IIIa was via different mechanisms [31]. GP Ib-mediated platelet adhesion is divalent cation-independent and is not inhibited by RGDS-containing peptide. On the contrary, platelet adhesion mediated by GP IIb/IIIa is inhibited by EDTA and RGDS-containing peptides.

2.3. Clotting Factors and Coagulation

Coagulation is initiated by two main mechanisms, the intrinsic or extrinsic pathways, and proceeds through a series of complex reactions that converge upon a final common pathway, causing the conversion of FGN to fibrin [32]. The plasma proteins involved in the blood coagulation are designated as “Factor” with a Roman numeral. Figure 2 illustrates the scheme of the interactions between the clotting factors involved in both the intrinsic, extrinsic and common pathways. Except for the contact phase in the beginning of the intrinsic pathway, calcium ion is required for most reactions of the coagulation process.

The intrinsic pathway starts with calcium-independent adsorption of Factor XII to the exposed, negatively-charged ECM molecules in a damaged vessel wall. Adsorbed Factor XII is converted to the activated Factor XIIa, which catalyzes the transformation of prekallikrein to kallikrein, and Factor XI to Factor XIa. Factor XIa then converts Factor IX to Factor IXa, which subsequently forms a complex with Factor VIII on the phospholipids surfaces of activated platelets to activate Factor X to Factor Xa. Factor VIII, an essential cofactor in the intrinsic activation of Factor X, requires pre-modification by thrombin to exert its physiological activity.

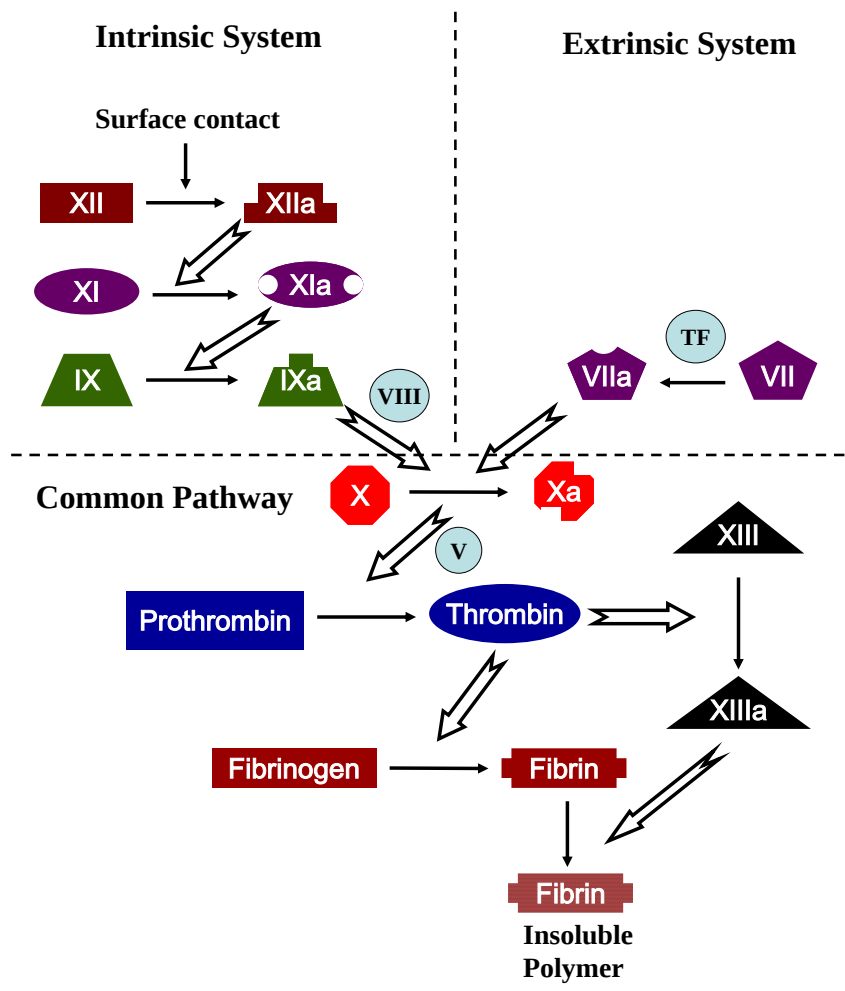


Figure 2. Mechanisms of the clotting cascade. The names of the clotting factors are deviated by the Roman numerals. Active enzymes in the cascade are designated by “a”. The factors in the circles represent co-factors.

The extrinsic pathway is initiated by the activation of Factor VII, which needs a cofactor tissue factor (TF). TF, a membrane-associated protein, is present in many tissues as well as activated macrophages and endothelial cells. When the flowing blood contacts the exposed subendothelial vascular structures upon vessel injury, TF becomes available for the extrinsic pathway. Activation of Factor VII starts with interaction with TF on a phospholipid membrane, and Factor VII is cleaved by a number of proteases in blood. Finally, the TF/Factor VIIa complex on the cell membrane converts Factor X to Factor Xa, initiating the common pathway.

The common pathway begins with the activation of Factor X by either the intrinsic or extrinsic pathway. The common pathway depends on the actions of activated platelets. Stimulated platelets can secrete cofactor Factor V, and express its receptors. Factor V attaches to the platelet membrane, and forms a complex with Factor Xa and calcium ions as the prothrombin activator. Factor Xa then converts prothrombin to the active enzyme thrombin.

The procoagulant role of thrombin is to cleave FGN into fibrin monomer and fibrinopeptides A and B, and to activate Factor XIII to Factor XIIIa. Fibrin monomers then polymerize to form a gel of long fibrin fibers, which is further covalently crosslinked into an insoluble fibrin clot (thrombus) by Factor XIIIa [33]. Within the clot, the fibrin plays a role in bridging plasma proteins and the platelets. The mature thrombus attaches to the blood vessel and prevents further bleeding.

In the process of coagulation, normally inactive clotting factors become enzymatically active by surface contact or proteolytic cleavage by other enzymes. The newly activated enzymes in turn stimulate many other normally inactive precursor enzymes in the next steps, so the reactions are quickly amplified through a series of sequences in the clotting reactions. Therefore, significant amounts of thrombin are produced in a short period of time.

Platelet aggregation and coagulation are further collaborated to ensure successful hemostasis. Aggregated platelets express negatively-charged membrane phosphatidylserine that accelerates two critical steps of blood coagulation: the activation of Factor X and the conversion of prothrombin to thrombin. The negatively-charged surface of the aggregated platelets thus serves as a site where thrombin can form rapidly. Thrombin also activates platelet directly, which then catalyzes the production of more thrombin. Activated platelets may also promote the activation of Factor XII and Factor XI.

2.4. Blood Flow

On injured subendothelium, vWF mediates platelet adhesion and aggregations. As described previously, vWF associated with injured vessel wall binds GP Ib on the platelet surface, which slows down the platelet's movement, thus making it to stay longer on the vessel surface [34]. The subsequent activation of the adhered platelet and GP IIb/IIIa renders the stronger binding of activated GP IIb/IIIa with surface-bound vWF and soluble FGN, leading to irreversible platelet adhesion and aggregation [34-39].

Beside platelets, plasma proteins and vessel surfaces, flow plays a pivotal role in hemostasis and thrombosis [37, 40]. In 1858, Rudolf Virchow proposed three general factors influencing blood coagulation in the cardiovascular system: the flow, the blood (coagulability) and the surface contacting blood (the thrombogenic nature), referred as "Virchow's Triangle" [41]. The convective transport of platelets and adhesive proteins from blood to the vessel wall is facilitated by increasing shear rates. Furthermore, shear rate can affect hemostasis and thrombosis by modulating the interactions between the adhesive proteins and platelets [42, 43]. Under high shear stress conditions, only vWF binding to GP IIb/IIIa supports platelet aggregation and thrombus formation [44]. On the other hand, FGN, which could mediate platelet aggregation under low shear force via its binding to GP IIb/IIIa, does not support platelet aggregation under high shear rates.

2.5. Fibrinolysis

After the damaged tissue is repaired, dissolution of the insoluble fibrin fibers is required to restore normal blood flow and facilitate the healing process in the damaged region. Fibrinolysis is mainly catalyzed by the enzyme plasmin, derived from the zymogen,

plasminogen, in plasma [45]. During the coagulation process, plasminogen adheres to fibrin clots and is incorporated into the thrombus. Plasminogen is activated to plasmin by the actions of plasminogen activators, tissue plasminogen activator (tPA) or urokinase, that may be present in blood or released from endothelial tissues [46, 47]. Activated plasmin is a non-specific serine protease capable of degrading the fibrin clot to dissolve the thrombus [48, 49]. The fibrinolytic process is summarized in Figure 3.

3. BLOOD-CONTACTING DEVICES AND MATERIALS

A wide range of materials, including metals, alloys, ceramics, natural and synthetic polymers, are extensively employed for blood-contacting medical devices. Among them, synthetic polymers are of particular interest because they can be easily combined with biomolecules chemically or physically to create biological functionalities. Moreover, the advantages such as low cost, easy fabrication, excellent mechanical properties (strengths, durability, and flexibility), versatile chemical characteristics, and relatively good hemocompatibility allow synthetic polymers to be extensively selected for blood-contacting medical devices [50].

The blood-contacting medical devices are categorized into transient (from few days to few weeks), intermit (up to one month) and long-term (more than one month) applications according to the blood-biomaterial contact duration [51]. Common transient blood-contacting devices include some types of catheters, guidewires, cardiopulmonary bypass, intra-aortic balloon pumps, and introducers, which are generally fabricated from polyurethane and silicone elastomers. The preparation of non-fouling surfaces which repel the blood adhesive components such as proteins, platelets and cells is an effective approach to treat transient blood-contacting devices. For example, hybrid polymer layer coating, hydrogel surface modification, and anti-adherent coatings are different types of anti-thrombogenic coatings to provide short-term solutions [52].

The devices in contact with blood up to 30 days, such as coronary-bypass oxygenators, central venous access catheters, coronary stents, and hemodialysis equipment, require a more stable anti-clotting coating. Polypropylene (PP), polyurethane (PU), polytetrafluoroethylene (PTFE), silicone elastomer and polysulfone (PSf) are commonly employed for the fabrication of intermit blood-contacting applications. One of the practical anti-coagulant methods is the immobilization of heparin which may prevent platelet adhesion and activation.

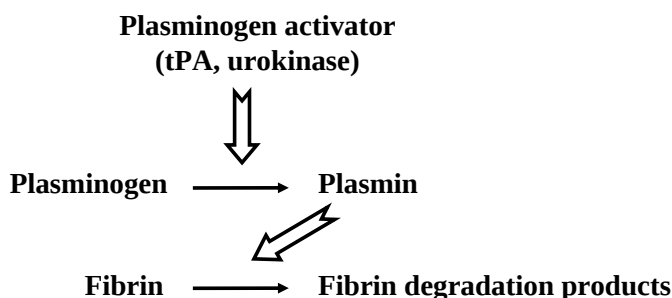


Figure 3. Schematic of the fibrinolytic process.

Chart 1. Categories of blood-contacting medical devices, the synthetic polymers used for their fabrication, and the examples of FDA approved products.

Devices	Materials	Trade Name (Manufacturer)
Catheters and cannulas	• Polyurethane (PU) • Silicone elastomer • Fluorinated ethylene-propylene (FEP)	• EOPA 3D[®] Arterial Cannulae (Medtronic Inc.) • Duraflo II Heparin Treated Femoral Cannulae (Baxter Bentley Laboratories) • TandemHeart Transseptal Cannula Set-EF (Cardiac Assist, Inc.)
Oxygenators (Cardiopulmonary bypass)	• Silicone rubber • Microporous polypropylene	• Heparin Coated Miniature Centrifugal Bypass Pump (A-Med Systems, Inc.) • Affinity Hollow Fiber Oxygenator with Trillium Biopassive Surfac (Avecor Cardiovascular Inc (Medtronic)) • Heparin Coated Extracorporeal Circuits (American Bentley)
Stents	• stainless steel • gold • TiN • Nitinol • PU • PTFE	• BX Velocity with Hepacoat Coronary Stent (Cordis Corporation) • Flype/HiFlype (Datascope Corp., (MAQUET Cardiovascular)) • Polyflex[®] Esophageal Stent (Boston Scientific Corporation)
Hemodialysis	• Polyethersulfone • Polyvinylpyrrolidone • Polycarbonate • Polyacrylonitrile • Cellulose	• Hemodialysis Dialyzers (Baxter International) • NxStage System One[™] (NxStage Medical, Inc.) • Hemodialysis Dialyzers (Kawasumi Laboratories)
Blood collection, blood bags	• Plasticized-PVC • Metallocene polyethylene (mPE) • ethylene-vinyl acetate (EVA) resins	• Venoject Ii Blood Collection Tubes (Terumo Corp) • Vacutainer Brand Blood Collection Syringe (Becton Dickson) • Ven-O-Vac Blood Collection Vacuum Tubes (Artefactos De Vidrio S.A. De C.V.)
Mechanical heart valves	• Ti • Ceramic • Nitinol • silicone + PU	• ATS Open Pivot[®] Bileaflet Heart Valve (TS Medical, Inc.) • On-X[®] Prosthetic Heart Valve, Model ONXA (Medical Carbon Research Institute (MCRI) • Mitroflow Aortic Pericardial Heart Valve (CarboMedics Inc.)
Synthetic vascular grafts	• PET • PTFE	• Gore Propaten Vascular Graft (W.L. Gore & Associates, Inc.) • InterGard Silver Collagen Coated and Heparin Bonded Vascular Prostheses (Datascope Corp., (MAQUET Cardiovascular)) • CryoArtery[®] Aortoiliac Artery, CryoArtery[®] Femoral Artery (CryoLife, Inc.)

For medical devices such as artificial heart, mechanical heart valve, and vascular grafts that will be staying in patients permanently for life extending purposes, biomaterials with excellent mechanical properties and stability such as silicone elastomer, PU, polyethylene terephthalate (PET), PTFE, and fluorinated ethylene-propylene (FEP) were frequently

utilized. The requirements for long-term usage of such blood-contacting medical devices are the inertness to immune system and the prevention of coagulation. Therefore the long-term efficacy is usually facilitated by mimicking the functions of the endothelium linings that can be executed by promoting the growth of an endothelium layer over the device surface, or by immobilizing bioactive agents directly onto the device surface [3].

The commonly used synthetic polymers for blood-contacting devices (Chart 2) will be discussed in this section in terms of the structures, physical-chemical properties, and how these materials have been modified to accommodate the requirements of hemocompatibility, respectively [53, 54].

3.1. Polyurethane (PU) and Polyether Urethane (PEU)

PU is a block copolymer consisting of polyurethane/urea “hard” segment and polyether or polyester “soft” segment [51]. The soft block in PU has glass temperature (T_g) much less than room temperature, presenting a rubbery character to the materials. On the other hand, the hard blocks with T_g above room temperature perform as glassy or semicrystalline reinforcing blocks. PU is frequently utilized for implantable devices such as blood filters, pacemaker lead insulation, catheters, heart valves, and artificial heart pump chambers for the hydrolytic resistance, long-term mechanical properties, stability, and blood compatibility [55, 56]. The applications of PU in blood-contacting devices range from artificial heart, heart valves, kidney components, surgical prostheses, catheters, blood vessels, to the assist devices including hemodialysis tubings, intravenous feeding kits and blood collection bags [57-59].

Blends of polyether and PU were created for their new properties such as water and solvent resistance, hardness and elasticity. For example, polyether urethane (PEU) was used in a variety of biomedical devices for its excellent physical and mechanical properties. Before contacting with blood, surface modification is required for PEU. For example, Biomer[®], a trade name for a segmented polyether urethane, has been shown to possess better blood compatibility than Silastic[®] and other materials [60]. It is also suggested that the blood compatibility of PEU is influenced by the relative surface concentration of the soft segments [61].

3.2. Polyethylene (PE) and Polypropylene (PP)

PE is the most well-known thermoplastics and the simplest vinyl polymer, made from ethylene monomers. Linear PE (high density PE, HDPE) is highly crystalline with better mechanical property than the branched one (low density PE, LDPE) which shows highly amorphous structure. PP, structurally similar to PE but with a methyl group attached on every carbon atom in the backbone chain, is also a thermoplastic polymer. Both PP and HDPE show high rigidity, chemical resistance and tensile strength and therefore are applied in medical devices for artificial hips, tubing for drains and catheters [62, 63].

Chart 2. The structure of polymers commonly used for blood-contacting medical devices

Polymer	Repeat units
Polyurethane	$\left[\text{H} - \text{N} - (\text{CH}_2)_x - \text{NH} - \overset{\text{O}}{\parallel} \text{C} - \text{O} - (\text{CH}_2)_y - \text{O} - \overset{\text{O}}{\parallel} \text{C} \right]_n^*$
Silicone rubber	$\text{—Si—O—} \left(\text{—Si—O—} \right)_n \text{—Si—}$
Polypropylene	$\left[\begin{array}{c} \text{H} \quad \text{H} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{CH}_3 \quad \text{H} \end{array} \right]_n^*$
Polyethylene	$\left[\begin{array}{c} \text{H} \quad \text{H} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{H} \quad \text{H} \end{array} \right]_n^*$
Polytetrafluoroethylene	$\left[\begin{array}{c} \text{F} \quad \text{F} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{F} \quad \text{F} \end{array} \right]_n^*$
Fluorinated ethylene-propylene	$\left[\begin{array}{c} \text{F} \quad \text{F} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{F} \quad \text{F} \end{array} \right]_n \left[\begin{array}{c} \text{F} \quad \text{F} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{F} \quad \text{CF}_3 \end{array} \right]_m^*$
Polyethylene terephthalate .	$\left[\text{O} - \overset{\text{O}}{\parallel} \text{C} - \text{C}_6\text{H}_4 - \overset{\text{O}}{\parallel} \text{C} - \text{O} - \text{CH}_2 - \text{CH}_2 \right]_n^*$
Polyvinyl chloride (PVC)	$\left[\begin{array}{c} \text{H} \quad \text{Cl} \\ \quad \\ \text{—C—C—} \\ \quad \\ \text{H} \quad \text{H} \end{array} \right]_n^*$

3.3. Silicone Rubbers

Silicone rubber, also named as silicone elastomer, is a flexible polymer with longer backbones consisting of silicon-oxygen linkages comparing with the basic polyethylene, accounting for flexibility. Besides, silicone rubber, a good insulator, resists to high temperature and can be operated from -55 to 300°C.

Silicone elastomers are applied clinically not only to blood-contacting implants such as heart valve and blood vessels, but also to plastic implants for chin, nose and outer ears. Due to its high oxygen permeability, silicone rubbers are popular materials for membrane oxygenators. However, it is important to pay more attention on the weight loads while silicone rubber is applied to medical uses for its low tensile strength [64].

3.4. Polytetrafluoroethylene (PTFE), Fluorinated Ethylene-Propylene (FEP), and Poly(Ethylene Terephthalate) (PET)

PTFE, FEP and PET are the most utilized materials for vascular grafts for functioning well in a high flow setting with cumulative patency rates [65, 66]. Commonly known as Teflon, PTFE is a compositionally simple polymer with a long and straight carbon backbone bonded to fluorine atoms. The combination of strong C-C and C-F bonding and the nonpolarity of fluorine atoms make PTFE extremely inert, thermally stable, lubricous, and very hydrophobic [67, 68].

FEP, a copolymer of tetrafluoroethylene and hexafluoropropylene, has a lower melting point than that of PTFE, causing easier processibility while maintaining the excellent chemical inertness and low friction characteristics [69]. Due to its highly transparent and resistant to sunlight, FEP is softer than PTFE and suitable for fabricating catheters.

PET (Dacron) can be polymerized from ethylene terephthalate monomer and is colorless with high transparency due to both amorphous and semi-crystalline compositions. PET is commonly employed in cardiovascular implants such as sewing rings of the mechanical heart valve prostheses, vascular grafts, cardiac patches and angioplasty balloons because of its excellent mechanical properties and moderate biocompatibility [70].

3.5. Polyvinyl Chloride (PVC)

PVC, a widely used thermoplastic polymer after PP and PE, is produced by polymerization of the vinyl chloride monomer. PVC is intrinsically hard, brittle and not blood-compatible, but the addition of plasticizers during processing can impart flexible character [71-73]. The advantageous properties of PVC include mechanical strength, chemical stability, simple fabrication, biocompatibility and sterilization performance, leading to applications in fabrication of lightweight, durable, non-breakable medical devices such as blood storage bags, tubings for extracorporeal circulation, and intravenous catheters [74-76], which also can be supplied in sterile packages. For long-term applications, PVC may cause problems because the plasticizers can be extracted by the body, while the plasticizers have low toxicities, resulting in less mechanical strength.

4. BLOOD-MATERIALS INTERACTIONS

The hemostatic mechanism, which arrests bleeding from injured blood vessels, may produce problematic or even life-threatening consequences when medical devices are placed in contact with blood. Compared to the normal unperturbed endothelium, all blood-contacting devices in use today generally suffer incompatibility with blood to some degree [77, 78], leading blood clotting at the site of the implant, continual shedding of thrombi, and depletion of platelets from the blood stream. Thromboembolic complication is a major cause of mortality and morbidity with cardiovascular devices. Many serious clinical complications on blood-contacting devices have been pointed out [41]:

- (1) small diameter vascular grafts (< 6 mm) suffer from early thrombotic occlusion;
- (2) embolic complications are frequently noticed with artificial hearts and catheters;
- (3) patients with mechanical prosthetic heart valves require life-long anticoagulation treatment;
- (4) blood damage is significant during hemodialysis and extracorporeal blood oxygenation.

These adverse events involve a complex set of inter-dependent reactions between (1) the biomaterial surface, (2) circulating platelets, and (3) the coagulation proteins. It is generally acknowledged that poor hemocompatibility of synthetic biomaterials is initiated from the instant formation of an undesirable and uncontrolled protein layer on biomaterials surfaces, once contacting blood. The adsorbed plasma proteins initiate platelet aggregation and blood coagulation cascade.

Circulating platelets recognize and adhere to surface-bound plasma adhesive proteins via platelet membrane integrins, which later initiates the activation of resting platelets and causes platelet aggregation. Among the plasma proteins, FGN, fibronectin, vitronectin and vWF have been shown to be capable of mediating platelet adhesion to artificial surfaces in their purified forms [79]. Nevertheless, FGN plays an important role in mediating platelet adhesion under static or shear conditions [79-83], while vWF plays a critical role in mediating platelet adhesion at high shear rates [82, 83]. Since FGN is present in plasma and is adsorbed on biomaterial surfaces in much higher quantity than the other adhesive proteins, the correlation between FGN adsorption and platelet adhesion has been focused in many studies.

Platelet adhesion to plasma-preadsorbed substrates under static condition was almost completely inhibited when the plasma was deficient of human FGN, while platelet adhesion was not affected by depletion of fibronectin, vitronectin, or vWF from plasma [79]. Furthermore, platelet adhesion was restored in a dose-dependent manner when the afibrinogenemic plasma was reconstituted with exogenous FGN [79, 80]. It is well known that FGN adsorption from native and diluted plasma to a variety of substrates exhibits an unexpected pattern, called the "Vroman effect", i.e. FGN adsorption reaches maximal at intermediate plasma dilution than from either native or very thin diluent [84-87]. It has previously been shown that platelet adhesion to Biomer[®] also reached maximal at intermediate plasma dilution [84]. The similarity in the patterns of FGN adsorption and platelet adhesion to Biomer[®] suggests a positively quantitative correlation between the amount of adsorbed FGN and platelet adhesion.

On the other hand, some studies indicated that platelet adhesion to FGN-coated surfaces should also depend on the conformation of the adsorbed FGN. Lindon et al. showed that although similar amount of FGN was adsorbed to various polymethacrylates, platelets retention on these substrates was varied [88]. In contrast, the binding of anti-FGN antibodies to the adsorbed FGN was varied with the types of polymethacrylates, suggesting that the adsorbed FGN possesses different conformation or orientation on the various polymethacrylates. The difference in conformation/orientation of adsorbed FGN may affect the exposure of the biologically active regions of the FGN molecule (e.g., RGDS or the C-terminal dodecapeptide of the γ chain), and thus cause platelet adhesion to vary. Another study also showed that no significant correlation between platelet adhesion and the amount of adsorbed FGN was found, while platelet adhesion was positively correlated to the binding of adsorbed FGN to three types of monoclonal antibodies which recognized the regions close to the platelet binding sites of FGN [89]. A partial least-squares calibration analysis indicated that the γ chain C-terminal dodecapeptide is the most important domain of adsorbed FGN in mediating platelet adhesion. Thus, platelet adhesion appears to be a bivariate function of adsorbed FGN, dependent on both the amount and the state of the adsorbed protein. Thomas Horbett at University of Washington proposed a mathematical model to describe the correlation between platelet adhesion and FGN adsorption under static condition [81]:

$$P = f_1(\Gamma_{FGN}) \times f_2(\Gamma_{FGN}) \quad (\text{Equation 1})$$

P represents the amount of platelet adhesion; Γ_{FG} presents the adsorbed amounts of FGN; f_1 is a function that platelet adhesion increases with increasing amounts of adsorbed FGN; f_2 , molecular potency, is a function that characterizes the fraction of the adsorbed FGN with a biologically active conformation capable of binding to GP IIb/IIIa.

It is important to note that platelets bind to surface-bound FGN via GP IIb/IIIa without pre-stimulation [13, 90-92], in contrast to that platelets do not bind to fluid phase FGN unless the platelets have first been activated with an agonist such as ADP or thrombin. This discrepancy suggests that surface-bound FGN is presented in conformation that is recognized by the unactivated GP IIb/IIIa. The concept of “substrate activation” arose from the above observation that the binding of resting platelets to the adsorbed form of FGN appeared to be stronger than that to the soluble form. The fact that adsorption of adhesion proteins to solid substrates enhances the biological activity of the surface-bound proteins is thought to arise from conformational changes in the adsorbed proteins that enhance the availability and potency of the cell-binding domains of the adhesive proteins.

Similar to the mechanism of platelet adhesion to injured blood vessels, the concept of Virchow’s triangle has been also adapted to explain platelet adhesion to artificial substrates. Although platelet adhesion to polystyrene [79] or polyurethane [81] under static conditions was not affected when the substrates were coated with vWF-deficient plasma, platelet adhesion at high shear rates (500 or 1000 s^{-1}) to several substrates preadsorbed with vWF-deficient plasma was greatly reduced in comparison with those preadsorbed with normal plasma, and was restored to normal when vWF was replenished [83]. On the other hand, FGN plays a critical role in mediating platelet adhesion at static and shear conditions, either low or high shear rate [82]. These studies concluded that both FGN and vWF play a role in mediating platelet adhesion under high shear conditions.

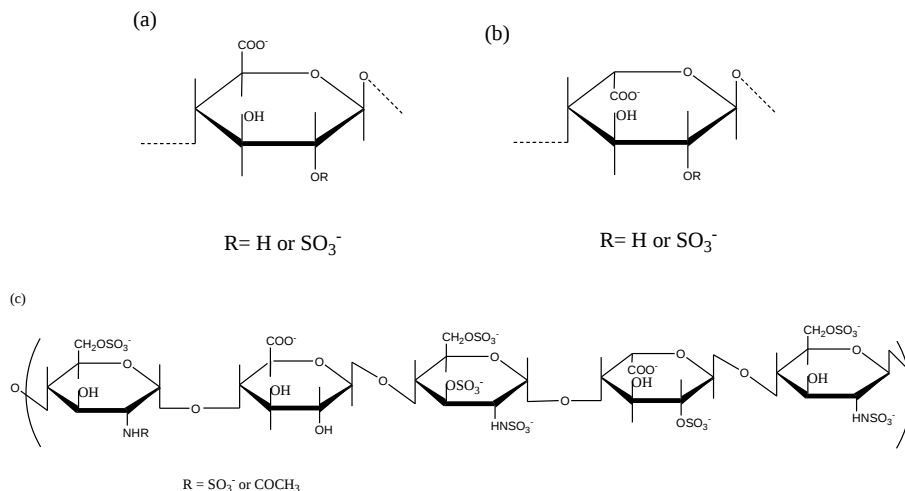


Figure 4. Structure of heparin: glucuronic acid (a), iduronic acid (b), and the antithrombin-binding pentasaccharide sequence of heparin (c).

Horbett later proposed a modified mathematic model for platelet adhesion to biomaterials under flow conditions [82]:

$$P = f_1'(\Gamma_{FGN}) \times f_2(\Gamma_{FGN}) + f_3(\gamma, \Gamma_{vWF}, \Gamma_{FGN}) \quad (\text{Equation 2})$$

The adsorbed amounts of vWF (Γ_{vWF}) is added into the modified equation; f_1' is a modified f_1 , to which the effect of shear rate on the binding efficiency of FGN is added; f_3 is a function that describes the synergistic effects of Γ_{vWF} and Γ_{FGN} on platelet adhesion under high shear rates (γ).

Platelet adhesion to biomaterials initiates subsequent platelet activation, which accelerates thrombosis by promoting thrombin formation and platelet aggregation. Platelet activation is accompanied with a morphological transition of adherent platelets from discoid to round, extension of pseudopodia, and spreading, which depends on the thrombogenicity of artificial surfaces. Spreading of adherent platelets on artificial surfaces appears to trigger platelet aggregation. Platelet activation upon material contact is recognized by intracellular signaling [93, 94], release of the contents of the α -granules and the dense granules, and shift of negatively-charged phosphorylserine from the inner to the outer face of the lipid bilayer [95]. Factor Va and Factor Xa can then bind to the negatively-charged platelet membrane and form the prothrombinase complex. Factor Xa in the prothrombinase complex can accelerate the conversion of prothrombin into thrombin much faster than Factor Xa in solution [96]. The enhanced production of thrombin further stimulates fibrin formation and platelet adhesion and activation [97], resulting in failure of blood-contacting devices. It is found that both FGN and vWF act together to stimulate platelet activation in vitro. Grunkemeier et al. showed that co-adsorption of FGN and vWF to substrates induced greater platelet procoagulant activity and spreading compared to adsorption of either FGN or vWF alone [98].

5. SURFACE MODIFICATION FOR IMPROVING BLOOD COMPATIBILITY

To resolve the complications encountered at the blood-biomaterial interfaces, researchers utilized various approaches to improve blood compatibility of biomaterials. These strategies can be classified into two major categories. The first one is to utilize the anti-coagulant mechanism of endothelium lining, applying biological anticoagulants such as heparin, heparin-like bioactive substances, albumin, prostaglandin and urokinase, and chemical modification. [71, 99]. The second one is to develop a non-fouling surface which resists protein adsorption and/or cell adhesion.

In this section, we will review several approaches based on the two major strategies for improving hemocompatibility of biomaterials.

5.1. Heparin

Heparin and heparan sulfate are among the most abundant sulfated glycosaminoglycans (GAGs) in the extracellular matrix of animal tissues. Both sulfated GAGs consist of alternating units of glucosamine with glucuronic acid (GLA) or iduronic acid (IDA) (Figure 4a, 4b), holding a high density of negative charges. Heparan sulfate contains a greater proportion of GLA, where heparin, with a molecular weight varying from approximately 5 to 25 kD, contains more IDA [100]. Heparan sulfate chains tend to present as proteoglycan components on the surfaces of endothelial cells and leukocytes, providing vascular vessels with a thromboresistant lining [101]. On the other hand, heparin is released from degranulating mast cells and can dissociate from protein core of the proteoglycan as free GAG chains.

The biological activities of heparin and heparan sulfate result mainly from the electrostatic interactions between heparin with versatile proteins including enzymes, lipoproteins, growth factors, chemokines, and extracellular matrix proteins [102, 103]. The anticoagulant property of heparin associates with its binding to the lysine residues of antithrombin III (ATIII). The conformational change in ATIII, induced by binding to a specific 5-sugar-residue sequence (ATIII-binding sequence) in heparin (Figure 4c), converts ATIII from a slow to an extremely potent inhibitor of the activated procoagulants, thrombin and Factor Xa [104, 105]. Factor Xa, the common product of the intrinsic and extrinsic pathways, is the most sensitive to inhibition by the ATIII-heparin complex. On the other hand, the anti-thrombin activity depends on heparin's size, requiring at least 18 sugar units to efficiently form an ATIII/thrombin/heparin ternary complex.

The efficacy of heparin coating on improving biomaterials hemocompatibility was first demonstrated on graphite surfaces in 1963 by Gott et al [106]. Since then heparin-coating technologies have been extensively developed for several blood-contacting devices such as cardiopulmonary bypass circuits [107]. The techniques to immobilize heparin include but not limited to the following methods: (i) electrostatic interactions via heparin's negatively-charged sulfate groups, (ii) covalent grafting, (iii) integration into a hydrogel network, and (iv) incorporation into a bulk polymer for controlled release. The literature on the techniques for heparin immobilization has been reviewed extensively elsewhere [108-110]. The easiest

way for heparin immobilization is attaching it to positively-charged surfaces via electrostatic interactions with the high negative charge density of heparin. However, the formation of multiple weak ionic contacts is needed in order to retain heparin at a positively-charged surface, so the exposure of the ATIII-binding sequence might be hindered, which prevents heparin from expressing its anticoagulant activity. Therefore, a heparin-immobilization technology which is able to present free ATIII-binding sequences on the surface may be beneficial to inhibit thrombus formation.

Several heparin-immobilization technologies have been commercialized as clinical blood-contacting medical devices such as cardio-pulmonary bypass, hemodialysis, hemoperfusion techniques, and vascular grafts (see a thorough review [110]). To date, the most successful heparin type of a blood compatible surface has been the end-point-attached heparin surface, that was developed by a Swedish professor Olle Larm [111]. Later, the research team was spun-off into a company, Carmeda Inc., and continued to develop this invention into technology (Carmeda Bioactive Surface, CBAS[®]) and bring to the market [112]. In this technology, reactive aldehyde groups are first generated at the reducing termini of the heparin chains. The aldehyde groups are then reacted with surface primary amines by formation of Schiff's bases, which then are reduced to stable, covalent bonds. Since heparin is attached at one end, the ATIII-binding sequences can protrude from the surface and specifically bind ATIII from the circulating blood (See Figure 5).

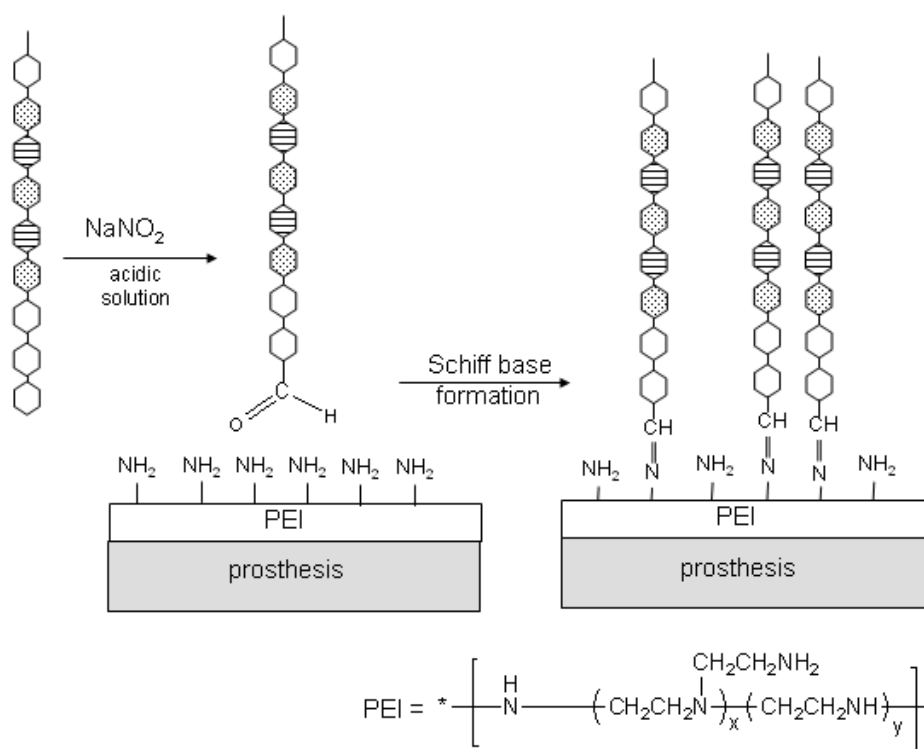


Figure 5. Schematic of heparin conjugation by CBAS[®]. After heparin chains are cleaved, the heparin segment bearing aldehyde groups are end-point attached to primary amines via Schiff base formation.

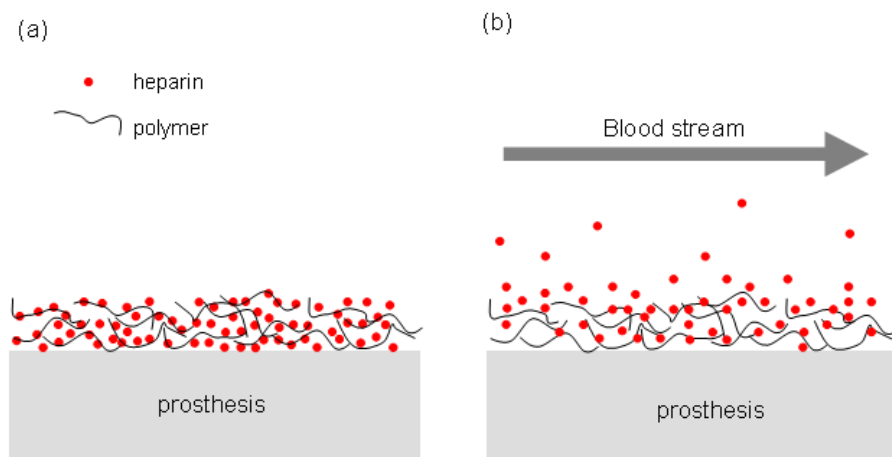


Figure 6. Schematic of Medi-Coat® technology: the entrapment of heparin in hybrid polymer layers on the surfaces of prosthesis (a), heparin is released after exposure to blood flow in a controlled manner (b).

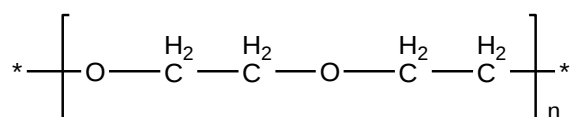


Figure 7. Schematic illustration of PEO structure.

Initial application of end-point immobilized heparin to extracorporeal circuitry showed the clinical potential of this method. It has been shown that heparin-coated extracorporeal circulation circuits were performed well in experimental animals or patients for extended periods of time (weeks or even months) without systemic anticoagulation and without any noticeable impact on the hemostatic mechanism [113, 114]. In 1987, Medtronic, Inc. first licensed this technique and developed into the first product – the MAXIMA oxygenator system. Since then, several other products including heparin-coated stent and vascular graft, have been introduced by Medtronic or other companies [112]. Nevertheless, the main disadvantages of the preparative process are time-consuming, difficult and expensive.

Although the anti-coagulant activity of heparin is generally attributed to its binding ability to ATIII, Weber et al. suggested that other mechanism involving the adsorption of other plasma proteins may be also important. They investigated the adsorption of plasma proteins to heparin-coating PVC tubing via the CBAS® technology and found that the heparin-coated surface altered the composition of surface-adsorbed plasma proteins such as fibronectin, FGN, C3 and high molecular weight kininogen (HMWK) [115]. The difference in protein adsorption may affect the selective uptake and cleavage of plasma proteins, platelet activation mechanisms and the subsequent release of coagulation factors, cytokines and adhesion proteins.

Another type of commercially available heparin-modified medical devices, based on Medi-Coat® technology which entraps heparin in hybrid polymer layers (Figure 6), also shows enhanced hemocompatibility [116]. The examples of applying Medi-Coat are the

coatings of the lumen and outside surfaces to modify polyurethane catheter tubing. The duration for releasing heparin coating can be ranged from days to months. Surface Solutions Laboratories Inc. has tried to developing or improving heparin coating technology in order to maintain longer heparin release over one month with simpler coating technique [115]. For the above applications associating bioactive molecules to modify the surfaces of medical devices, the ultimate time-determining procedure is to precede clinical studies for meeting regulation approvals which can be costly and time-consuming [71].

5.2. Hydrophilic Surfaces

It has been proposed that a surface with low interfacial free energy has low driving forces for protein adsorption [7]. Many neutral, hydrophilic polymers, including natural and synthetic, such as agarose, dextran, methylcellulose, poly(ethylene oxide)(PEO), polyacrylamide, show small tendencies towards protein adsorption [117, 118]. Among these polymers, PEO has attracted most attentions (Figure 7). PEO-presenting surfaces are viewed as an important class of biomaterials for a wide range of applications, such as blood-contacting devices, drug delivery systems, contact lenses, intraocular lenses, catheters, immunoassays, and biosensors.

Several mechanisms have been proposed to explain the protein-resistant property of PEO surfaces. Some of these theories are related to the unique PEO-water interaction properties. It is postulated that the structure of PEO is similar with water, so the strong hydrogen bonding between water and the ether oxygen atoms is formed, resulting in a highly connected arrangement of tetrahedrally coordinated water molecules around PEO [119]. It is suggested that two or three hydrogen-bonded water molecules are needed for basic hydration of each ether unit of PEO. Therefore, PEO is completely soluble in water at room temperature in all proportions [120]. On the other hand, other structure-related polymers such as poly(methylene oxide) and poly(propylene oxide) are water insoluble at common conditions [120].

The hydrophilicity and unique water-solubility properties of PEO render the polymer chains exhibiting considerable flexibility or high mobility in aqueous solution [121]. The dynamic behavior of PEO in aqueous environment has been investigated by nuclear magnetic relaxation, electron spin resonance, and the measurement of dynamic moduli [122-124]. PEO, owing to lack of charges and big side groups, appears to be very flexible compared with polymers with bulky side chains (steric hindrance) or polyelectrolytes (electrostatic hindrance). A rapid mobility of the hydrated PEO chains on a surface reduces the protein contact time, thus decreasing the possibility of protein adsorption [125]. Furthermore, the protein-resistant property of PEO may be explained by the theory of steric stabilization effect, including volume restriction and excluded volume [126-128]. Briefly, a repulsive force develops when a protein approaches the PEO surface. It appears that PEO surfaces in water have high mobility and a large excluded volume compared to the less water-soluble or insoluble polymers, thereby minimizing protein adsorption. A thorough review of the blood compatibility of PEO can be found [129].

PEO surfaces can be prepared by a variety of strategies, including physical adsorption, bulk modification, covalent coupling, and graft copolymerization. The simplest and easiest, but probably least stable method for PEGylation of a surface is to adsorb PEO or PEO

copolymers directly to a substrate [120]. Only PEO with a molecular weight higher than 100 kDa can be effectively adsorbed on hydrophobic surfaces [129]. On the other hand, PEO copolymers consisting of one adsorbing block, acting as an anchor to substrata, and the PEO motif, providing resistance to protein/cell attachment, are generally used. For example, PEO is copolymerized with polypropylene oxide (PPO) as PEO-PPO-PEO triblock, that can be attached to hydrophobic substrates (i.e. polyethylene [130] and polydimethylsiloxane [131]) via hydrophobic interactions between the PPO segments and the substrata. Polystyrene (PS) surfaces modified by PEO-PPO triblock copolymer showed minimized protein adsorption [132, 133], and blood interactions [134, 135]. On the other hand, the grafting of PEO to polyelectrolytes such as poly(L-lysine) (PLL) [136, 137], poly(ethylene imine) [138], or poly(acrylic acid) [139], facilitates PEGylation to charged surfaces via electrostatic interactions. For example, PLL-g-PEG spontaneously adsorbs onto negatively charged surfaces such as SiO₂, TiO₂ and Nb₂O₅ [136]. Nevertheless, the main pitfall for such approach is that the adsorbed copolymers do not stay on the surface permanently.

Bulk incorporation of PEO into the commonly used blood-contacting polymers provides an advantage over PEO coatings. In such copolymers, PEO is present throughout the bulk and surface. It is suggested that the biocompatibility of these copolymers will be retained even when subjected to surface abrasion or erosion [140]. The prepared copolymers are then fabricated into blood-contacting devices. As early as in 1960s, Lyman prepared PEO copolymers with PET or segmented PU for medical applications such as dialysis membranes [141, 142]. Among the polymers used in blood-contacting devices, segmented PU has been the most commonly studied PEO-based materials. Brash and coworkers showed that plasma protein adsorption to the segmented PU containing PEO as the soft segment was significantly lower than that using PPO [143-146]. Goodman et al. also demonstrated that platelet spreading and activation on PEO-containing segmented PU were minimal in comparison with the PU incorporated with other macroglycols [15, 147, 148]. Other studies also indicated that PEO-containing PU attracted less thrombin and fewer platelets than those composed of PPO or poly(tetramethylene oxide) in vitro [61, 148]. Furthermore, platelet retention was further reduced with increasing molecular weights of PEO.

In spite of in vitro success of PEO-incorporated block copolymers, it is not guaranteed that similar performance will be seen in vivo. Nevertheless, PEO-containing block copolymers have several potential pitfalls in medical applications. The surfaces may present significant amount of the non-PEO hard segments, which are usually highly thrombogenic [149, 150]. The PEO segments do not exist as pendant chains, which restricts the chain mobility of PEO. Furthermore, the mechanical properties of PEO-copolymers are sometimes not strong enough for biomedical applications [151].

Covalent conjugation of PEO to a substrate may be a better strategy. Conjugation of PEO or its derivatives on the surface of a polymeric substrate is more stable and maintains the mechanical strength of the bulk materials. For the substrates containing reactive functional groups such as hydroxyl, carboxyl or amine groups, PEG can be coupled directly to such substrates via suitable chemical reactions [152]. For example, PEO with a terminal carboxyl group can be coupled to the hydroxyl groups of cellulose dialysis membranes via ester linkage [153, 154]. It has been shown that the dialysis efficiency and blood compatibility of the PEO-modified dialysis membranes were improved by such strategy. Cyanuric chloride-activated PEO can be conjugated onto amine-containing surfaces. Desai and Hubbell showed

that protein adsorption and platelet adhesion to PEO-modified PET were decreased for about 50% and 90%, respectively [155].

For chemically inert surfaces such as PE, PP, and PTFE, plasma discharge or gamma irradiation treatment can be applied to create surface free radicals for PEG grafting [156]. Plasma glow discharge techniques have been used to covalently conjugate to alkyl PEO block copolymer surfaces [157, 158]. FGN adsorption was largely reduced on the PEO-grafted PE surfaces. Wang et al. demonstrated that grafting of PEO on PET substrates by argon plasma discharge greatly reduced the adhesion, aggregation and morphological changes of platelets [159]. Small oligo-ethylene glycol molecules such as triglyme or tetraglyme can also be deposited on substrates as PEO-like films by glow discharge plasma deposition [160-162]. Fibrinogen adsorption was reduced to an ultra-low level on the tetraglyme-deposited substrates [160, 163]. Platelet adhesion and thrombin generation by adherent platelets were greatly reduced on tetraglyme-coated materials such as glass, Biospan, and PET [160]. Such approach can be also used to treat the luminal surface of small diameter tubing, which form is widely used in catheters, and vascular grafts [161].

Although PEO has been widely applied to biomaterials applications to prevent non-specific protein adsorption and cell adhesion, the major pitfall of PEO-presenting surfaces is rapid auto-oxidation of this polyether, especially in the presence of oxygen and transition metal ion [164]. PEO decomposed after exposure to air at 45°C or 20°C for 1 week or 1 month, respectively. Furthermore, the hydroxyl groups of PEO can be oxidized enzymatically to aldehydes and acids *in vivo* [164]. Decomposition of PEO renders the attachment of proteins and cells to the substrates, and reduces the utility of PEO surfaces for long-term applications.

5.3. Phosphorylcholine-Based Polymers

Since the late 1970s phospholipids that make up the cell membrane have attracted much attention from biomaterials researchers due to their potential to improve biocompatibility of medical devices. Many cell types have an asymmetric lipid composition in the cellular bilayer membranes. For example, the inner cytoplasmic surface of platelets and erythrocytes consists of a larger proportion of negatively-charged phospholipids such as phosphatidylserine, while phosphatidylcholine (PC), a zwitterionic lipid, is the major component of the outer layer [165]. For example, PC is consisted of 88% of the head groups in the outer membrane of erythrocyte [166]. Zwaal et al. demonstrated that the phospholipids from the inside surface of the erythrocyte cell membrane in essence would cause clot formation when exposed to blood, while the lipids constituting the outer layer appeared to be non-thrombogenic [167]. It was previously shown that upon procoagulant activation of platelets, the negatively-charged phospholipids on the cytosolic layer such as phosphatidylserine flip to the extracellular face [168], providing a high affinity surface for assembly of Factor VIIIa/Factor IXa (Tenase complex) and Factor Va/Factor Xa (prothrombinase complex), which then accelerates blood clotting [96, 169]. On the other hand, phosphatidylcholine, which possesses an equal number of both positively and negatively charged groups and thus maintains overall electrical neutrality at the physiological condition, is inert in coagulation assays. It is logical to hypothesize that a surface mimicking the external phospholipid membrane of cells possesses non-thrombogenicity [170, 171].

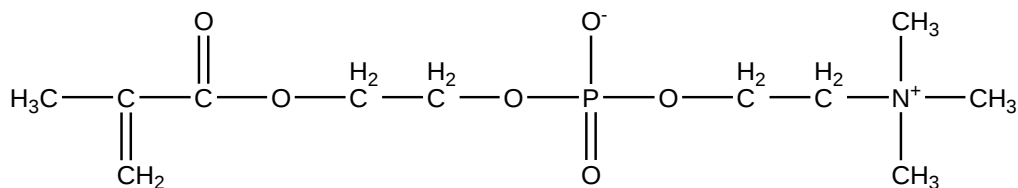


Figure 8. Schematic illustration of methacryloyloxyethyl phosphorylcholine (MPC).

The early challenge of applying phosphorylcholine in surface modification was to develop a biomimetic surface coating. Nakabayashi developed a technology based on synthesis of a methacrylate molecule having a phosphorylcholine group that can be copolymerized with other conventional methacrylates to create copolymers for preparation of reliable non-thrombogenic surfaces for blood-contacting devices. The structure of methacryloyloxyethyl phosphorylcholine (MPC) is shown in Figure 8. However, early advancement had been hindered by impurity of the MPC compounds, resulting in the formation of heterogeneous surfaces with mixtures of raw polymers, intermediates, and the desired structures. Ishihara [172] later improved the synthesis route to enhance the yield and the purity of MPC. Therefore, various copolymers of MPC with other acrylate monomers such as styrene, *n*-butyl methacrylate (BMA), *n*-hexyl methacrylate, and *n*-dodecyl methacrylate (DMA) can be easily synthesized via free-radical polymerization, and provide a wide range of properties [170, 173, 174]. Some researchers suggest that MPC with long-chain alkyl methacrylates such as BMA and DMA could mimic more closely the structure of naturally occurring membrane lipids, which should benefit the biocompatibility of MPC-modified surfaces [170].

Blood compatibility of poly(MPC) was initially tested by passing unheparinized human blood through a column that was packed with poly(methyl methacrylate) (PMMA) beads coated with copolymers of MPC and methacrylate [175]. Impressively, no platelet deposition was observed on the beads coated with 30 mole% MPC copolymer with methacrylate after 15 minutes of contact. Several similar studies indicated that a copolymer with above 30 mole% of MPC is sufficient to suppress platelet adhesion and activation [173, 175, 176]. Due to its excellent anti-coagulation ability, MPC-based copolymers have been used extensively in a wide range of medical device applications including biosensors, drug carriers, soft contact lenses, vascular stents, and urological devices [170, 177, 178].

The mechanism of the anticoagulant property of the deposition of MPC on a surface was first hypothesized by Nakabayashi in late 1970s and was ascribed to the strong affinity of MPC to phospholipids from plasma [179]. The speculative scheme of low thrombogenicity of MPC surfaces starts with adsorption and accumulation of blood phospholipids on the MPC surface. The adsorbed phospholipids rearrange to a layer mimicking cellular membrane. Plasma proteins fail to adsorb to such “biomimetic membrane”, and blood cells may recognize it as epithelium of natural vessel and flow through without interactions. Many studies verified that MPC polymers could reduced protein adsorption [175, 180-182], and the extents of the increase in adsorption of phospholipids and the decrease in protein adsorption were correlated the MPC contents [175]. Nevertheless, the above mechanism cannot be the only explanation in the low protein adsorption of MPC polymers, since several in vitro protein assays indicated that MPC polymers remain resistant to protein adsorption without the

presence of free plasma phospholipids [183]. Other work proposed the importance of the high free-water fraction associated with the polymer surface [184]. The free-water fraction is considered as one of the important factors in the blood compatibility of hydrophilic polymers. High water fraction gives MPC polymers hydrogel-like property, and could prevent proteins from significant conformational change and irreversible adsorption.

The techniques of immobilization of MPC to blood-contacting surfaces are similar to those of PEO immobilization. MPC can form copolymers with conventional methacrylates. Many strategies utilize the properties of co-polymerized monomers to incorporate phosphorylcholine moieties on a surface. Here some commonly used techniques of immobilization of MPC and their clinical applications are introduced.

A straightforward way to immobilize MPC copolymers to a substrate is physio-adsorption. MPC can be simply adsorbed or coated to a substrate by copolymerization with a monomer. For example, copolymers of MPC with alkyl methacrylates are suitable for adsorption as a monolayer on hydrophobic surfaces. The coated MPC films are reported as extremely stable with excellent biocompatibility. It is suggested that the success in hemocompatibility of such strategy mainly depend on the hydrophobicity of the substrates, while the length of the alkyl chain in alkyl methacrylates appears to have little impact [173]. Coating of MPC/lauryl methacrylate copolymer on blood filtration devices significantly decreased blood clotting compared to the uncoated filters [185]. Similarly, the hemocompatibility of filtration devices made of non-woven PET fibers was greatly improved by coating of copolymers of 2-methacryloyloxyethyl phosphorylcholine and 2-methacryloyloxyethoxyethyl phosphorylcholine [186]. A coronary stent coated with a phosphorylcholine-based polymer, which had been implanted in a human patient for 6 months, was retrieved and confirmed that the original MPC polymer coating possessed long-term stability *in vivo* [177].

Blending MPC copolymers with other conventional polymers used in medical devices has been proved as an easy and effective way to improve the blood compatibility of the base materials used in medical devices, such as PE, natural rubber, PU, and PSf [187-189]. Ishihara et al. modified PSf by blending of MPC copolymer with n-butyl methacrylate or n-dodecyl methacrylate in PSf [190]. Protein adsorption on the membrane composed of the blends of PSf/MPC copolymer was significantly decreased with an increase in the composition of the MPC content in the copolymer. The number and the degree of activation of the adherent platelets was also suppressed by the modification with the MPC polymer [190]. Small-diameter (2 mm) vascular prostheses, prepared from a polymer blend composed of segmented PU and MPC copolymer, were implanted in rabbit carotid arteries to evaluate the improvement of blood compatibility. Results indicated excellent anti-thrombogenic properties within 8 weeks [173]. Mixture of segmented polyurethane and poly(MPC-co-2-ethylhexyl methacrylate) was dip-coated on small diameter vascular grafts made of porous polyester woven fabrics [191]. The patency of the modified vascular grafts was evaluated in an *ex vivo* rabbit artery model. The control samples fabricated by pristine PU were occluded within 90 min, while the MPC-incorporated vascular grafts remained open for four weeks [192]. Although the blending method showed improvement on blood compatibility, the physical properties of bulk materials are reduced.

Surface grafting has been accepted as a stable and effective approach for the surface modification of biomaterials. Various chemical approaches can be applied to surface conjugation of phosphorylcholine (PC). Grafting of MPC copolymers has been accomplished

on various substrates through conventional free-radical polymerization with different initiation methods [193-197]. For example, Ishihara et al. used photoinduced polymerization technique to graft MPC onto PE membranes [196]. The PC-modified membranes showed significant reduction in protein adsorption and platelet adhesion compared to the unmodified PE [196]. Recently, Iwata et al. polymerized MPC on silicon wafers via surface-initiated atom transfer radical polymerization (ATRP) [198], that can control the length of polymer chains precisely [199]. They found that the poly(MPC) brush greater than 5.5 nm was effective to reduce protein adsorption and cell adhesion on these surfaces compared to the untreated substrates [198].

Another common approach to conjugate phosphorylcholine onto substrates is to synthesize a reactive derivatives of PC to react with a specific substrate. An addition of a thiol group on a PC molecule renders it attaching to a gold substrate using the self-assembling method [200]. PC monolayer was shown to reduce FGN adsorption and platelet adhesion compared with the untreated control. Durrani et al. prepared a series of reactive derivatives of phosphorylcholine which react with hydroxyl groups and acid chlorides on various substrates [201]. Biocompatibles Internations Plc., also synthesized a range of PC derivatives for attaching these PC derivatives to artificial surfaces (PC TechnologyTM) [202]

5.4. Zwitterionic Surfaces

Although MPC polymers have been shown successful in preventing protein adsorption and platelet adhesion, the tendency of the phosphoester group to be hydrolyzed [159] may impair the long-term stability of MPC polymers. Besides, MPC is moisture sensitive and not easy to synthesize and handle [173]. The inhibitory effects of PC on protein adsorption and cell adhesion are suggested to be due to the zwitterionic nature of the PC head groups carrying both positive and negative charges. The electrically neutral PC molecules at the physiologic pH are capable of binding a significant amount of water molecules, which prevents protein adsorption. Therefore, researchers suggest that molecules such as phosphobetaine, sulfobetaine, and carboxybetaine (Figure 9) whose zwitterionic structures similar to that of PC, i.e. both cationic and anionic groups are on the same monomer residue [203], are potentially excellent candidates to prevent protein adsorption and cell adhesion [204-206]. Compared with MPC, sulfobetaine methacrylate (SBMA) is easier to synthesize and handle.

SBMA-based polymers are applied to blood-contacting devices in a way similar to MPC copolymers. It has been shown that copolymers of poly(SBMA) or poly(carboxybetaine methacrylate) reduced platelet adhesion as compared to the controls [207, 208]. Nevertheless, some studies suggested that such copolymers possess less anti-fouling ability than MPC polymers [209, 210]. On the other hand, some researchers suggest that a surface with uniform and highly-packed sulfobetaine molecules should be superior in anti-fouling. It has been shown that uniform poly(SBMA) brushes formed by surface-initiated ATRP created a superlow fouling surface (e. g., FGN adsorption < 0.3 ng/cm²) [211].

So far, these polymers based on zwitterionic monomers have not been applied to biomedical devices as widely as MPC polymers. Many related studies are focused on fundamental investigation on some model surfaces such as gold, glass, silicon wafers and metals. The in vivo applicability of such materials needs further evaluation.

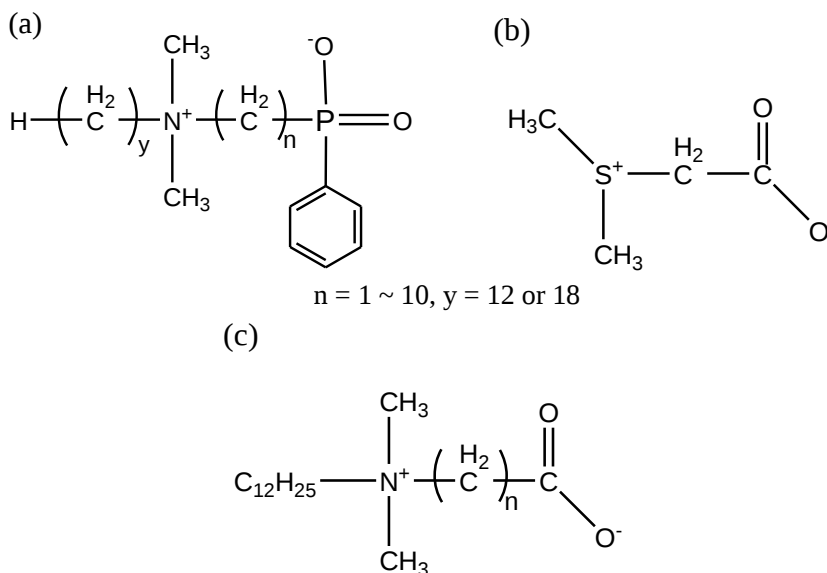


Figure 9. The schematic structure of the three kinds of betaine: phosphobetaine (a)[224], sulfobetaine (b), carboxybetaine (c)[225].

5.5. Clot-Dissolving Surfaces

Besides prevention of protein adsorption and platelet adhesion, one strategy is to resolve the problem after thrombus formation. John Brash at McMaster University in Canada proposed a concept that a surface that is capable of selectively adsorbing plasminogen from blood may dissolve the formed thrombus. Plasminogen contains lysine-binding sites in the loop-like structures known as kringle I and IV regions [212, 213]. The binding of plasminogen to the lysine residues in the fibrin promotes localization of plasmin during fibrin degradation [214-216]. Plasminogen-binding surface can be developed based on such intrinsic affinity of plasminogen for the lysine with free ϵ -amino groups. This concept was first proved by the experiments that lysine-conjugated polyurethanes and silica glass adsorbed plasminogen firmly from plasma [217, 218]. Furthermore, tissue plasminogen activator (t-PA) possessing one lysine-binding site [219, 220] also binds to lysine residues in fibrin. The interactions between plasminogen, t-PA, and exposed carboxy-terminal lysines on the surface of fibrin form a complex, converting plasminogen to plasmin in a manner that is at least 1000-fold more rapid than the conversion of plasminogen to plasmin in blood [221, 222], and facilitating fibrinolysis. Several studies indicated that strong "plasmin-like" activity was found on the lysine-derivatized substrates with free ϵ -amino groups following exposure to plasma and t-PA [212, 218, 223]. Clot lysis by these surfaces was demonstrated, suggesting that the ϵ -lysine surfaces have the potential to cleavage fibrin. [212]

Brash further developed a blood contacting surface that possesses both resistance to nonspecific protein adsorption and clot lysing properties. A PU surface was modified with PEG and lysine. Nonspecific protein adsorption was greatly reduced on these surfaces while significant amount of plasminogen was adsorbed from plasma. Such surfaces were able to dissolve plasma clots formed nearby [226].

6.CONCLUSION

In spite of the general success with the currently used blood-contacting medical devices, problems such as adverse effects of using anti-coagulants, degeneration of tissue, mechanical failure, postoperative infection, and induction of blood clots still remain. Among the complications, coagulation and thrombus formation are the major reasons for the failure, so there is a great demand for the improvements on the blood compatibility for biomaterials and medical devices.

Tremendous efforts, as presented in the content of this review, were made to understand and improve the blood compatibility of medical devices from different aspects. Hematologists and biochemists have interpreted the thrombus formation mechanisms by identifying the roles of biomolecules in the clotting cascades and the various coagulation pathways involved. On the other hand, biomaterial scientists, based on the rationales of anti-thrombogenicity and clinical necessities, focus on developing bioactive, non-fouling, and biomimetic medical devices by modifying the bulk or surfaces of biomaterials. Nevertheless, the universal non-thrombogenic biomaterial has not yet been developed. The necessities of better understanding of blood-material interactions and new invention/technology of biomaterials science are still required for future development of blood-contacting devices with excellent hemocompatibility.

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Chapter 6

BIOCOMPATIBILITY OF DENTAL AND MEDICAL MATERIALS

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ABSTRACT

A number of biomaterials, organic or synthetic, are used in the various fields of medical or dentistry. Among them are the suture materials, osteosynthesis plates and screws, endosseous implants, bone substitutes, endodontic, restorative, prosthetic materials, and others. All of them are put in contact with biological tissues, deeply or superficially and most of them remain for lifetime. For this reason, all these materials should be carefully analyzed by means of biocompatibility tests, in order to evaluate materials' biological behavior. In this regard, the goal of this chapter is to present the biocompatibility of some biomaterials such as bioactive bone substitutes (ceramics, bioactive glasses and polymers) titanium endosseous implants, metal and polymeric osteosynthesis devices, restorative and endodontic materials based on our current research with the field.

INTRODUCTION

Since antiquity mankind experiments the use of extra-body materials to be in contact with human biological system, from aesthetic to therapeutic purposes. Some of these materials were, and currently are used superficially in body as skin and teeth, while others are used

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deeply in the organism, in substitution to organs and tissues. The increasing knowledge on human system and tissues associated with technology advances has permitting the development of new biomaterials, which are extensively tested in order to evaluate their biological behavior. Considering the number of available biomaterials, the focus of this chapter is to discuss on the most used biomaterials in dentistry and medicine, as well as the current researches on biocompatibility tests.

BIOMATERIALS IN DENTISTRY AND MEDICINE

Williams (1987) defined biomaterial as a "*nonviable material used in a medical device, intended to interact with biological system*". Nowadays, innumerable biomaterials are available for medical use in order to substitute or improve organs and tissues, such as cardiovascular system, skeletal, and organ prostheses, among others. Dentistry is a branch of medicine divided into specialties that routinely employs a large spectrum of biomaterials, most of them intended to spend a life-time in superficial or deeply contact with oral tissues, as in medicine, causing different biological responses. Considering the particularities of the mouth as the facility of the innumerable exogenous agents to be in direct contact with oral mucosa and teeth, extra care should be taken in relation to the indication of the material used. Comprehensively, some biomaterials are used and indicated for both areas, due to their fundamental basis. Technological advance has permitted the development of new and high quality materials, and along with the demands of governmental health departments, a number of biocompatibility tests are required. For a better understanding, biomaterials will be divided into their composition and applied specialties.

Metallic Biomaterials

Metallic devices are extensively used in medicine and dentistry especially in the treatment of bone injuries in the form of a combination of different metals compounding the alloys. Stainless steel, commercially pure titanium (CP – ASTM F67) and extra-low interstitial (ELI) Ti-6Al-4V alloy (ASTM F136) (Brunski, 2004) are the preferred metals for osteosynthesis and implant devices. Although they present good clinical results, titanium has been the metal of choice because of its high biocompatibility (Krischak et al., 2004). The results presented by Branemark et al. in late 1970's attesting a 10-year result of endosseous integration for dental rehabilitation, strongly increased the interest on this metal, including its biological effects.

Titanium is known to be inert to human organism, mainly due to its high corrosion resistance. However, local release of titanium ions in bone and soft tissue from osteosynthesis devices (Figure 1), as well as from endosseous implants, has already been observed by many researches (Schliephake et al., 1993; Torgersen et al., 1995; Jorgerson et al., 1997; Acero et al., 1999). Body fluids may participate of this process, but also, the manipulation and insertion of the devices can also result in small debris that can be left in local tissues, and possibly systemically delivery to distant organs by macrophage cells. Accumulation of titanium in organs such lungs, kidneys, liver (Schliephake et al., 1993; Bessho et al., 1995)

and lymph nodes (Weingart et al., 1993) has been confirmed, by means of light and electronic microscopy, energy-dispersive X-ray and atomic absorption spectrophotometry analysis; however, no clinical manifestation was detected and the biological relevance of these findings remained unclear.

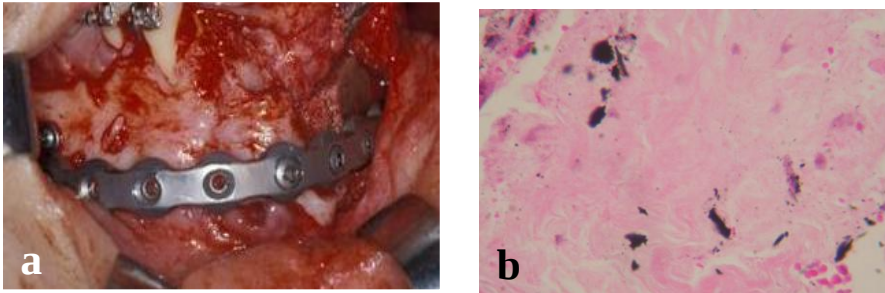


Figure 1. a) Titanium plate and screws for fixation of a mandibular fracture; b) dark granules observed in a retrieved fragment of connective tissue in close contact to the plate, suggesting metal release (Gently offered by Dr. Hugo Nary Filho).

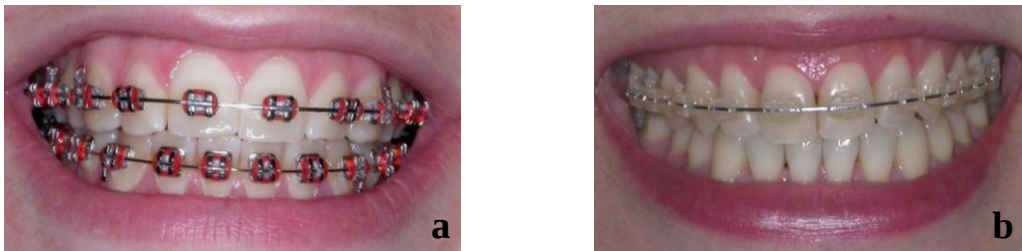


Figure 2. Metallic orthodontic brackets in *a*, and the esthetic acrylic brackets in *b*.



Figure 3. Extensive amalgam restorations maintain close contact with oral mucosa and fluids.

In order to collaborate for the elucidation of the presence of titanium particles in organs, we recently evaluated titanium miniplates *in vivo* by the single cell gel (comet) assay (Piozzi et al., 2009). Albinus Wistar rats received titanium miniplates and screws in their tibia and after experimental periods of 30, 90, and 180 days, their lungs, liver and kidneys were retrieved for analysis and no genotoxicity was pointed out. Absence of genotoxicity was also observed in a previous *in vitro* analysis of eluates from titanium endosseous implants by the same assay (Ribeiro et al., 2007d). These results, along with the optimal clinical results, especially in dental implantology field, confirmed by recent 20-year follow-up studies (Ekelund et al., 2003; Lekholm et al., 2006; Astrand et al., 2008), still maintain titanium as the metal of choice for bone tissue.

Interest in the amount of metal ion intake in direct contact with oral tissues has grown in the last decades due to the high local application of metallic components in orthodontics and restorative dentistry. Fixed orthodontic appliances, like brackets, bands, and archwires usually made of stainless steel, nickel-titanium, nickel-cobalt alloys, can also release metal ions in the presence of oral fluids. Faccioni et al. (2003) showed that nickel and cobalt released from fixed orthodontic appliances can induce DNA damage in oral mucosa cells *in vivo*. Similar results were found using gingival fibroblasts *in vitro* (Tomakidi et al., 2000). In order to decrease such damages, ceramic and polycarbonate brackets are alternatives (Figure 2). However, no significant cytotoxicity differences are noted when comparing metallic and non-metallic brackets according to Mockers et al. (2002) and Pereira et al. (2009).

Another metallic source which is in direct and prolonged contact with oral mucosa is found in amalgam tooth restorations (Figure 3). Eritematous lesions in the mucosa close to this material are observed in some patients, known as oral lichenoid lesions (OLL). Koch and Bahmer (1999) found 15 patients out of 19 sensitive to inorganic mercury presenting OLL adjacent to amalgam fillings. After fillings removal, the lesions of 13 out of 15 inorganic mercury-sensitized patients with OLL (86.7%) healed or significantly improved. Similar results were obtained by Issa et al. (2005), with complete healing of OLL in 42% of 39 patients who had their amalgam restoration replaced. However, not only local alterations can be caused by amalgam. In addition, systemically free mercury causing skin hypersensitivity reactions have been reported (Fardal et al., 2005).

Bioceramics

Millions of bone fractures occur every year worldwide. Among them, 10-30% result in non-unions (Zimmermann & Wentzensen, 2007), others exhibit delayed healing (Einhorn, 1998); thus indicating that, in some cases, bone cannot heal through its normal wound healing response. In addition, impairment of bone regenerative growth and remodeling can be caused by other clinical situations involving bone loss, such as various diseases or tumor resection (Gautier et al., 2005).

In this context, there is a critical need for technologies that are able to enhance bone healing (Damien et al, 2004). Autogenous cancellous and compact bone grafts have been used in the reconstruction of these defects because they do not cause immunogenic reactions, and present osteogenic cells' release growth factors that contribute to repair of the defects (Hings et al., 2004). Despite the advantages offered by autogenous bone grafts, they require a prolonged surgical time, the amount of bone that can be safely harvested is limited and they

can cause pain and morbidity. For these reasons, alternative biomaterials, so-called bone substitutes, have been investigated (Yoo et al., 202). Undoubtedly, dental implantology highlighted this necessity, since oral rehabilitation using endosseous implants, as the terminology states, depends on the presence of enough bone tissue for treatment. It explains the increased number of researches found in this specialty, and the indication of some particular biomaterials for this purpose. However, no matter the etiology of the defect, major bone reconstructions frequently depend on autogenous bone grafts. Otherwise, whenever possible, smaller defects tend to be treated using biomaterials in order to avoid disadvantages already mentioned.

Bioceramics represent a broad range of inorganic/nonmetallic compositions (Hench; Best, 2004), including hydroxyapatite, bioactive glass, calcium phosphate, and others, that have been extensively used in particulate form, acting as fillers and scaffold for bone defects reconstruction promoting a tri-dimensional matrix in order to stabilize and maintain the shape of the filled area. It also permits and supports cell migration and angiogenesis, resulting in new bone formation during repair (Spector, 2008; Walsh et al., 2008). Each alloplastic biomaterial presents specific chemical and physical characteristics, which directly influence rate and time of bone formation. Although they are osteoconductive in the majority, bone substitute biomaterial should not only substitute bone, but act in a way that its complete absorption occurs, permitting the formation and its replacement by a new bone tissue. This response is mainly dependent on its origin, shape, and porosity characteristics (Spector, 2008).

Synthetic hydroxyapatite (HA) has been one of the most frequently used ceramic because of its chemical composition similarity to human bone, non toxicity, high chemical stability, and absence of inflammatory or antigenic reactions (Hing et al, 2004). One of the advantages of hydroxyapatite is that its microstructure can be controlled to promote the formation of pores that can allow the migration of blood vessels and bone tissue into the material (Silva et al, 2005). This material is known as a slowly biodegradable material with high osteocompatibility and bone binding capability, and its resorption rate is relatively slow compared with the rate of new bone formation (Ricci et al., 1992). Artiz et al. (2004) stated that the low-lasting presence of bovine HA incorporated to bone, creates a dense cancellous network, by the strengthening of bone tissue mass.

Many studies have investigated the effects of hydroxyapatite on bone metabolism and as a substitute for autogenous bone grafts in the reconstruction of bone defects. Silva et al. (2005) evaluated the repair of calvarial bone defects in rats comparing autogenous bone grafts and porous bioceramic discs of hydroxyapatite/phosphate cement mixture. They found that by week 24, the defects filled with bioceramic material were almost completely closed as a result of the joining of ceramic fragments and the neoformed bone tissue. Moreover, Orr et al. (2001) found an increase in the modulus of elasticity of the bone defects in rabbits implanted with a synthetic hydroxyapatite material 26 weeks post surgery.

In oral and maxillofacial reconstruction, bovine HA represents one of the most important biomaterial for bone reconstruction aiming rehabilitation with endosseous implants (Olson et al., 2000; Schlegel et al., 2003; Hallman & Nordin, 2004; Dahlin et al., 2009). An interesting result was presented by Schelegel et al. (2003) comparing maxillary sinus augmentation using autogenous bone grafts and bovine HA in beagle dogs. After 90 days, a slight improve in bone-implant contact and volume in the bone grafts group; however, after 180 days, these results inverted, with a decrease in bone volume of sinuses treated with bone grafts.

Chemically pure tricalcium phosphate (TCP) is another material currently used for bone grafts due to their similarity to the mineral phase of bone and excellent osteoconductivity. It is available in two distinct forms: α -TCP, modified by high temperatures, over 1.125°C, and the β -TCP, modified by low temperatures, under 1.125°C, being α -phase more soluble than β -phase. When α -TCP is used as bone substitute, its dissolution occurs before new bone formation, resulting in a fibrous tissue. Modifying material phase and increasing porosity, degradation and resorption rates can be adapted to follow bone regeneration (Wiltfang et al., 2002). Former types of TCP, due to their heterogeneous compounds, were often contaminated with foreign phases, leading to different degradation times (Merten et al., 2001).

β -TCP has biodegradable properties and it is also considered to have high osteocompatibility and to be resorbed at a higher rate than HA. Dias et al (2004) showed that 12 week post surgery, β -TCP implants were surrounded by new bone tissue in tibia defects of rabbits. Study comparing the effects of HA and β -TCP implants in parietal bone in rats showed that, at 2 weeks, osteogenesis from the parietal bone was observed around both materials, and new bone had attached directly to the surfaces of HA and TCP. However, the volume of new bone around HA was larger than that around TCP. Also, the authors found that there was no remarkable change in the amount of remaining HA, but that of TCP was decreased (Fujita et al., 2003). Orztuck et al. (2006) evaluated the effects of hydroxyapatite/tri-calcium phosphate (HA/TCP) implants and demineralised bone matrix (DBM) on bone consolidation in rats. The authors found that 10 weeks after implantation, the use of DBM alone demonstrated improved healing on radiographic and histological studies compared to other groups and the control group. Interestingly, no difference was found between the HA/TCP treated group and the control group.

As previously mentioned, bone substitutes represent a welcome alternative to autogenous bone grafts considering oral rehabilitation using dental implants, especially in bone cavities as maxillary sinuses, since they avoid an extra surgical site for graft retrieval, but mainly because they sustain bone defects while healing, permitting the insertion of the implants before complete bone formation (Schelegel, 2003). The necessity for understanding the effects of these materials on cells and tissue has leading researches to search for relevant information from molecular biology techniques. The identification of growth factors involved in osteoblasts differentiation and synthesis, as well as in vascularization, contributes to the comprehension of different healing processes. Zerbo et al. (2005), in biopsies retrieved from human maxillary sinuses after 6 months reconstruction with β -TCP, observed bone growth into material porous, as well as soft connective tissue. Positive immunostaining for Runx-2/Cbfa-1 (core-binding factor-1), a transcription factor required for activation of osteoblast differentiation, osteopontin and bone sialoprotein in (pre) osteoblast, young osteocytes and in the connective tissue cells around or in the particles of β -TCP suggested that these cells were following osteogenic differentiation pathway. Our group also analyzed the presence of Runx2/Cbfa-1 and VEGF (vascular endothelial growth factor) in maxillary sinuses augmentation of rabbits using HA (Bio-Oss[®]) and β -TCP (Cerasorb[®]). Our results showed a stronger Runx2/Cbfa-1 immunostaining in HA specimens than β -TCP group, but similar rates of VEGF in both groups, in an attempt to contribute with the elucidation of biomaterials behavior (unpublished data). Inert implants, like some metals, make bone formation possible permitting a mechanical adhesion to the material, called biological fixation. However, the considered bioactive materials, such as the cited biomaterials in this section, induce a

biological response in the interface area, creating a chemical adhesion with bone, called bioactive fixation, due to the presence of calcium and phosphate. The surface of these materials induces a biologically active carbonated layer of hidroxyapatite that creates an adhesive interface with the tissues. In addition, researches have shown the stimulation of osteoblastic differentiation *in vitro* by local rise in calcium and phosphate ions (Sugimoto et al., 1993).

Another promising treatment is the use of bioactive glasses as bone graft substitutes due to their ability to bond and integrate with living bone by forming a biologically active bonelike apatite layer on their surfaces (Hench & Polak, 2002; Hench, 2003). These materials are considered to be a rapid deposition of a layer of hydroxycarbonate apatite (HCA) on the surface of the silica gel as a result of exchange between calcium and silica ions when bioactive glass came into contact with living tissues (Hench, 2003). Entirely inorganic reactions, previously described by Hench (2003), lead to rapid growth of HCA bone mineral on the glass surface and to a rapid proliferation of new bone within a bone defect site, a process termed osteoproduction. The size, crystal habit, orientation and composition of the HCA phase are equivalent to that of the mineral phase of living bone. This phase provides binding site for osteoprogenitor cells attachment, proliferation and differentiation; hence cell function is favored and osteoid matrix is generated (Hench, 2003). Indeed, collagen fibrils are incorporated within the mineralized layer of newly forming bone, as demonstrated by electron microscopy of the glass interface bonded to bone (Davies & Baldan, 1997).

One of the most used bioglasses for bone graft is the Bioglass[®] 45S5, a silica-based melt-derived glass (45% SiO₂, 24.5% Na₂O, 24.5% CaO, 6% P₂O₅). This biomaterial has been known for many years as the most bioactive composition among numerous bone-bonding glasses (Hench, 2003). It was first introduced in the early 1970's by Hench and since then, it has been reached many clinical applications, including the repair of periodontal bone defects, maxillofacial defects reconstruction, spinal surgery and bone replacement (Hench, 2003; Scarano et al., 2006).

Many works have proved that Bioglass[®] 45S5 has good bone bonding properties and a stimulatory effect on osteogenesis (Reilly et al., 2007; Nandi et al., 2008; Vargas et al., 2008). Human bone cell culture over the surface of Bioglass[®] 45S5 discs demonstrated enhanced formation of bone nodules, which is a three-dimensional structure composed of mineralized extracellular matrix and cell aggregates with organizational complexity similar to natural bone grown *in vivo* (Xynus et al., 2000). In addition, the ionic products of Bioglass[®] 45S5 induced an increase in both human osteoblast proliferation and expression of insulin-like growth factor II (IGF-II), a potent osteoblast mitogenic growth factor (Xynus et al., 2000b). Indeed, the mechanism for *in-situ* tissue regeneration involves upregulation of seven families of genes that control the osteoblast cell cycle, mitosis and differentiation (Hench et al., 2004).

Despite its well-known stimulatory effects on osteogenesis, the use of monolithic Bioglass[®] 45S5 for bone engineering applications has been limited mainly due to its relatively poor mechanical properties (Vallet-Regi, 2001; Dieudonne et al., 2002). Considering this important matter, our research group has developed nucleation and growth thermal treatments to obtain a novel fully-crystallized bioactive glass-ceramic of the quaternary P₂O₅-Na₂O-CaO-SiO₂ system (Biosilicate[®], patent application WO 2004/074199). Crystallinity significantly changes the fracture characteristics of glasses leading to glass-ceramics with improved mechanical properties. On the other hand, the introduction of crystalline phases could, in principle, decrease the bioactivity (Vallet-Regi, 2001). But, interestingly, *in vitro*

experiments demonstrated that Biosilicate[®] promotes enhanced bone-like matrix formation in comparison to its parent glass and to Bioglass[®] 45S5 in an osteogenic cell culture system (Moura et al., 2007).

This important finding stimulated our group to progress the understanding of the effects of this fully crystalline glass-ceramic on osteogenesis. We investigated the *in vivo* biological performance of the Biosilicate[®] as a potential biomaterial for bone augmentation restoration. In an experiment investigating the effects of Biosilicate[®] on bone defects in tibias of rats, the three-point bending test revealed a significant increase in the maximum load at failure and stiffness in the animals treated with Biosilicate[®] in comparison with the control group. Also, the Biosilicate[®] group was superior to Bioglass[®] 45S5 in the histological analysis. Biosilicate[®] presented higher bone volume and number of osteoblasts in the defect area (Granito et al, 2009).

Polymers

Many types of polymeric materials are widely used in biomedical devices, including silicones, acrylic, and resins, among others. Depending on their composition and arrangement they can be found in form of gel, soft, malleable, and hard materials, being absorbable or not, resulting in a wide variety of applications in different biological conditions.

Synthetic polymeric suture materials present the best tissue response, contributing to an improved healing process, replacing organic sutures known to cause important local inflammatory reactions in a circumstance where it should promote the exactly contrary effect. Polyglactin acid, polyglycolic acid, polyglycaprone, and polytetrafluorethylene are examples of polymers used as sutures, and their advantages over organic material is the lack of antigenicity and low local inflammatory response. Also, their physical characteristics, e.g. mono or multifilamentous, contribute to influence tissue repair quality. It is important to emphasize that no suture material is specially developed for oral tissues application, and their use in oral surgery is adapted from medical practice, taking into consideration the particular conditions of the mouth. Okamoto et al. (1990) studied the effects of different suture materials after tooth extractions, and observed that better responses were obtained with nylon, whereas silk and cotton produced considerable delay of socket healing. Different synthetic sutures were analyzed by Nary Filho et al. (2002) in subcutaneous tissue of rats: polyglycaprone 25, polyglactin 910, and polytetrafluorethylene. Although all materials induced satisfactory healing, a better response was achieved using polyglycaprone 25, highlighting the influence of its physical monofilamentar characteristic for the favorable results, also beneficent to avoid bacterial adherence, as shown by Banche et al. (2007).

Absorbable polymers are also being used for the confection of osteosynthesis devices, composed of high molecular weights macromolecules, including polydioxanone (PDO), polyglyconate, polyglycolic acid (PGA), poly-L-lactide acid (PLA), and PGA-PLA copolymers (Pietrzak et al., 1996). They were idealized in order to avoid undesirable effects caused by metallic components, as previously mentioned, and thinking of a non-necessity of a second surgical procedure if removal of the osteosynthesis is necessary. In addition, it does not interfere in imaging exams. Some disadvantages to be considered are the high-cost of the material and the long permanence of the devices until it is complete absorbed, which contraindicates its application when bone reconstruction aims rehabilitation with endosseous

implants. The prolonged absorption process extends inflammatory reaction, which could interfere with the implants stabilization (Matsumoto et al., 2005).

An example of non-absorbable polymer is the high-density porous polyethylene (HDPP), indicated for craniofacial contour recovery, for esthetic and functional purposes. Long-term results and minimum complications caused by HDPP have already been reported elsewhere (Rubin, 1983; Yaremchuck, 2003; Menderes et al., 2004; Gosau et al., 2006; Gürlek et al., 2007). Besides being a highly biocompatible material, its physical characteristics like the presence of pores and their specific size are equally important. Klawitter et al. (1976) stressed the relevance of size pores around 100-250µm for optimal tissue in growth, in a canine model. The optimal biological behavior of HDPP led some authors to test its possible osteogenic capacity. Bilkay et al. (2004) compared the biochemical levels of alkaline phosphatase and osteocalcine of hydroxiapatite, HDPP, and bone collagen positioned in tibial periosteal sacs of rabbits in the periods of 1, 2, 4, and 8 weeks. Despite results pointed out HDPP as presenting the highest osteogenic ability, it is known that it was its appropriate architecture that permitted better tissue response and ingrowth, improving periosteum osteogenicity. Recently, Oliveira et al. (2009) investigated the expression of Cbfa-1 and VEGF in bone repair using HDPP fixed in the mandible of rabbits. The results demonstrated a moderate Cbfa-1 expression at initial periods established in this study after HDPP implantation. In the same way, VEGF displayed immunoreactivity in the interface area, as depicted by immunopositive cells on the capillary walls, demonstrating that HDPP is able to adequately permit osteoblasts differentiation as well as neovascularization into the material.

Acrylic devices intended to remain inside the mouth during prolonged periods are also an issue of concern, as partial or total dentures. Humidity and warm environment created between oral mucosa and dental prostheses favors some micro-organisms growth, as the fungi *Candida*, suggested as one of the etiological agents in the development of a specific type of oral lesion named chronic denture stomatitis, mainly affecting hard palate (Figure 4) (Emami et al., 2007; Coco et al., 2008). The existence of certain micro-organisms of oral microbiota able to metabolize alcohol to acetaldehyde, known as a carcinogenic agent is emphasized by Meurman and Uttamo (2008). Among them, *Candida* is pointed out, since it possesses the enzyme alcohol dehydrogenase, common in poor oral health population, and one of the conditions found in oral cancer patients. Such evidences on these fungi led us to study the cells from oral mucosa affected by chronic denture stomatitis and to evaluate them in order to observe cell cytotoxicity and the presence of micronucleus (Matsumoto et al., 2009). A total of 23 affected adult patients had their exfoliated palatal lesioned mucosa taken, and after established technique steps, the cells were stained by Feugen-Fast Green method, for micronucleus visualization. No significant micronucleated cells were noted; however, nuclear alterations such as karyorrehexis, pyknosis, and karyolysis, indicating cytotoxicity, were found. Nevertheless, authors remember that no single test is capable of detecting all genotoxic agents, and that a more detailed judgment on the genotoxic potential of chronic stomatitis on oral cells, a battery of tests may be necessary.

Dental Materials

Tooth and periodontium are highly specific structures of oral cavity, constituted of particular tissues that create unique microenvironments. In this way, the biomaterials

indicated to be in contact with these tissues are equally specific, and will be designated as "dental materials" in this section, although some of them can find applications in fields other than dentistry.

Carious lesions are still an issue of concern in developing countries. Its progression, due to enamel and dentin destruction by bacterial products, can reach dental pulp, the living part of the tooth, leading to its partial or total elimination. If inflammation products invade periodontal tissue, i.e. periodontal ligament, through apical foramen, periapical lesions as granulomas and cysts may invariably arise. The main purposes of endodontic therapy are to eliminate microorganisms in root canals and fillings in order to restore dental function, and for this, some aggressive antimicrobial solutions are indicated. During endodontic practice, these solutions can be in contact with oral tissues due to an inadequate manipulation, or in contact with periodontal tissues, via apical foramen. Also, if periapical lesions are persistent after endodontic therapy (Figure 5), a surgical approach is performed for its removal, and a material is used to seal apical foramen, which will remain in permanent contact with periodontal tissues.



Figure 4. Erythematous chronic stomatitis in hard palate, identified by the presence of diffuse red spots.

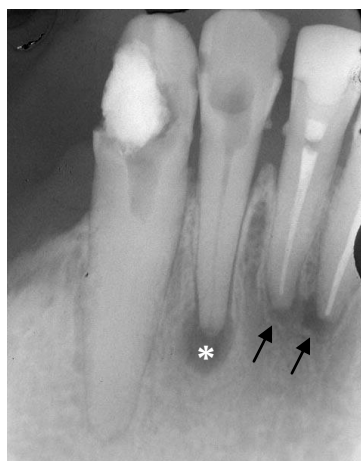


Figure 5. Periapical lesions (*) in a pulp compromised tooth and endodontically treated teeth (arrows).

To best of our knowledge, only a few studies on putative genotoxicity of endodontic agents have been conducted and those report with divergent findings. Huang et al. (2001) have argued that zinc oxide eugenol-based root sealers do not induce DNA damage in oral carcinoma cells *in vitro*. However, they have detected a dose-dependent increase of genotoxicity when cells were exposed to resin-based sealers. These data are largely of the same magnitude as those reviewed by Geurtsen et al. (1997). Later, Tai et al. (2002) found that N2-, AH26-, and AH-Plus-based root canal sealers exhibited genotoxicity by causing DNA single-strand breaks. The same findings were encountered by Schweikl et al. (1998a) by means of AMES test. However, Huang et al. (2002a,b) have demonstrated that resin-based sealers such as AH26 and AH-Plus sealers exhibited cytotoxicity and a dose-dependent increase of DNA damage *in vitro* assessed by single cell gel (comet) assay. More recently, this research group has evidenced no apparent genomic damage induced by root canal sealers (Huang et al., 2004).

In human dental pulp fibroblasts exposed to phenolic compounds, potent antimicrobial agents, no DNA breakage was detected (Chang et al., 2000; do Céu Silva et al., 2003). Conversely, these same substances were able to induce unscheduled DNA synthesis in a concentration-dependent manner on SHE cells (Hamaguchi and Tsutsui, 2000). Recently, we investigated whether formocresol, paramonochlorophenol or calcium hydroxide could induce DNA damage in mouse lymphoma cells and primary human fibroblasts by the single cell gel (comet) assay *in vitro*. These materials are widely used in endodontic practice to eradicate bacteria and consequently to produce root canal disinfection. Our data demonstrated that the single treatment with formocresol, paramonochlorophenol and calcium hydroxide at concentrations of 20, 40 and 80 µg/mL did not induce strand breaks in DNA (Ribeiro et al., 2004d). Similar results were observed in CHO cells after exposure to formocresol, paramonochlorophenol and calcium hydroxide adjusted to 100 µg/mL (Ribeiro et al., 2005a). In fact, it has been reported that chlorophenol failed to promote unscheduled DNA synthesis in the presence or absence of exogenous metabolic activation (Hagiwara et al., 2006), although cresol induced unscheduled DNA synthesis in the presence of exogenous metabolic activation (Hamaguchi and Tsutsui, 2000). By contrast, statistically significant differences in the frequencies of sister-chromatid exchanges were observed in SHE cells treated with chlorophenol or formaldehyde (Miyachi and Tsutsui, 2005) and chromosomal aberrations were induced by formocresol, with chlorophenol being a significant chromosomal aberration inducer in the presence of exogenous metabolic activation only (Hagiwara et al., 2006). Besides cresol, formocresol is also composed of formaldehyde that is a known genotoxic and cytotoxic substance (Casanova et al., 1994; Lovschall et al., 2002). The primary genotoxic effect seems to be the formation of DNA-protein crosslinks (Merck and Speit, 1999). In a recent study designed by Ramos et al. (2008), formocresol induced DNA-protein cross-link and an increased frequency of micronucleus in bone marrow cells *in vitro*.

Our research group studied gutta-percha solvents, such as chloroform and eucalyptol focusing on genetic damage *in vitro*. Our results demonstrated that both compounds do not promote DNA breakage in CHO or mouse lymphoma cells after single exposure (Ribeiro et al., 2006h; 2007b). In the same way, some radiopacifiers, widely used in clinical dental practice to permit radiographic visualization of structures and materials that are not radiopaque in nature, were also analyzed. Human peripheral lymphocytes obtained from healthy volunteers were exposed to barium sulphate (BaSO₄), zirconium oxide (ZnO₂) and bismuth oxide (Bi₂O₃) at final concentrations ranging from 1 to 1000 µg/mL. The results

pointed that all tested compounds did not induce DNA breakage in human peripheral lymphocytes (Braz et al., 2008).

Taken together, it seems that some endodontic compounds induce genetic damage as depicted by genotoxicity results. Therefore, the use of these compounds should be cautious in dentistry.

To ensure normal growth control and integrity in DNA molecule, cells have developed many strategies to manage stress. However, a failure on some of these defense mechanisms may lead to the development of degenerative diseases such as cancer. Nowadays, it is well established that exogenous agents can modify the cellular DNA, along with other cellular components. In this way, it would be useful to know whether, and to what extent, these antimicrobial endodontic compounds possess synergistic and/or antagonist effects with known genotoxins present in our environment. Thus, we have performed assays to investigate the ability of formocresol, paramonochlorophenol and calcium hydroxide to modulate the genotoxic effects induced by the oxidatively damaging agent hydrogen peroxide and the alkylating agent methyl methanesulfonate *in vitro*. The addition of methyl methanesulfonate in increasing concentrations to cells incubated with formocresol, paramonochlorophenol or calcium hydroxide did not contribute to genetic damage (Ribeiro et al., 2006f). Similar findings were observed with respect to hydrogen peroxide for all endodontic compounds evaluated (Ribeiro et al., 2006f).

In the 1990's, mineral trioxide aggregate was developed as a root-end-filling material. A number of biocompatibility studies has been conducted either *in vitro* or *in vivo* showing that mineral trioxide aggregate has good sealing ability and tissue healing properties. Nevertheless, genotoxicity data were needed to complete risk assessment of mineral trioxide aggregate. Herein, we have investigated whether regular and white mineral trioxide aggregate can induce DNA damage *in vitro* by means of mouse lymphoma and CHO cells. The results showed that the materials tested did not induce DNA strand breaks at concentrations ranging from 1-1000 µg/mL as depicted by the single cell gel (comet) assay (Ribeiro et al., 2005b; 2006b,c). These findings confirmed and extended the data already published showing a good biocompatibility of mineral trioxide aggregate.

Nowadays, studies have compared mineral trioxide aggregate with Portland cement. The findings suggest that they seem almost identical macroscopically, microscopically and by x-ray diffraction analysis, and contain the same chemical elements. This information indicates that Portland cement has the potential to be used as a less expensive root-end-filling material in dental practice. We have observed the absence of genotoxicity in rodent neoplastic cells exposed to regular and white Portland cements at concentrations ranging from 1-1000 µg/mL. To verify the genotoxicity in ordinary human cells, we exposed lymphocytes to these compounds *in vitro* (Ribeiro et al., 2006c). Mineral trioxide aggregate at concentrations up to 1000 µg/mL did not induce genotoxic effect (Braz et al., 2006). Furthermore, no DNA damage was found for ordinary and white Portland cements (Braz et al., 2006). In summary, these data are consistent with the notion that mineral trioxide aggregate and Portland cements had no genotoxic effect.

Since glass ionomer cements were first introduced in the early 70s by Wilson and Kent (1972), they have been used extensively in dentistry as restorative materials and adhesives for composite restorations, once its major advantage lays on fluoride ion gradually liberation at tooth surface. Their usage also includes prosthetic and orthodontic devices. Some studies

have demonstrated that glass ionomer cements are able to induce DNA damage in various test systems such as the bacterial UMU-test, eukaryotic DNA synthesis inhibition test, *in vivo* alkaline filter elution technique as well as sister chromatid exchange test in human lymphocytes (Heil et al., 1996; Stea et al., 1998; Muller et al., 2003). However, negative results were also observed under *in vitro* experiments (Schweikl et al., 1994; 1998b). Such discrepancies have been attracting attention to new studies on genotoxicity of these cements. Data obtained in our laboratory using CHO cells demonstrated that the powder from Ketac Molar displayed genotoxicity only at the maximum concentration tested (100 µg/mL) (Ribeiro et al., 2006e). Similarly, liquid from Vitrebond at 0.1% dilution caused an increase of DNA damage (Ribeiro et al. 2006g). These data are in accordance with those described by Kleinsasser *et al.* (2004; 2006) who found only a small genotoxicity induced by 2-hydroxyethyl methacrylate (HEMA), a component of liquid Vitrebond. No significant DNA damage was noted at concentrations possibly relevant to *in vivo* situations (10^{-4} M) (Spahl et al., 1998) Nevertheless, studies reported that HEMA induced apoptotic death, probably affecting the intrinsic apoptotic pathway as well as generating oxidative stress (Paranjpe et al., 2005; Becher et al., 2006; Lee et al., 2006; Samuelsen et al., 2007). In conclusion, these results support the rationale that some glass ionomer cements induced DNA migration detected by single cell gel (comet) assay as a sign for limited genotoxic effects in higher concentrations. High cytotoxicity was evidenced either *in vitro* or *in vivo* (De Souza Costa et al., 2003; Lan et al., 2003; Souza et al., 2006). With the highest levels of DNA migration being combined with elevated cytotoxic effects, therefore, a low *in vivo* noxious activity could be observed. Further studies must be conducted with isolated substances of these glass ionomer cements in order to clarify the action mechanism.

Concluding Remarks

Undoubtedly, continuous advances of biotechnology and researchers efforts in testing and adapting innumerable materials developed to interact with biological systems represent an enormous contribution for human health, and most of the therapeutic possibilities now available wouldn't be possible without these materials. Our aim was to present some of these biomaterials in their specific applications, as well as their relation to cells and tissues; however, besides many others were not mentioned in this chapter, their relevance in medicine and dentistry are undisputable.

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Chapter 7

ENHANCING REMINERALIZATION OF SUBSURFACE ENAMEL LESIONS WITH FUNCTIONALIZED β -TCP

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ABSTRACT

In an attempt to further the anticaries benefits afforded by fluoride, we have developed a unique functionalized tricalcium phosphate (fTCP) and explored its remineralization potential in several dental models. fTCP was found to be stable in NaF aqueous solutions of 500, 950, and 5000 ppm F, and is the first form of bioavailable calcium that is stable with fluoride in an aqueous and single-compartment environment. Using an *in vitro* pH cycling regimen emulating actual events transpiring in the oral cavity, we demonstrated that the baseline remineralization of softened enamel with topical solutions of 500, 950, and 5000 ppm F can be substantially improved when combined with fTCP. Using both Vickers and Knoop microhardness measurements, the reversal of white-spot lesions was attributed to both surface and bulk remineralization effects with fTCP providing statistical improvement over the base fluoride solution. However, in a single-step dental model, fTCP was found to limit fluoride uptake. This result was inconsistent with multiple-step regimens and previously reported work, but furthers our understanding of the influence fTCP has on enamel. Because fTCP does not compromise fluoride bioavailability and is not limited to boosting remineralization through elevating fluoride uptake alone, we explored the role fTCP has with enamel tissue and fluoride. Based on our results, we conclude fTCP intertwines synergistically with both fluoride and the enamel tissue in a sophisticated manner that produces superior remineralization of the subsurface lesion relative to fluoride alone.

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1. INTRODUCTION

The mammalian tooth is an interesting structure comprising layers of soft inner media and a hard outer surface. The cartoon shown in Schematic 1 illustrates the multilayer structure. The pulp chamber maintains the blood and nerve supply and is protected by the dentin, which attenuates shock and pressure experienced during mastication or in the event of a traumatic event. The inorganic mineral content in dentin is quite low compared to enamel with calcium weight fractions in dentin is about 28% lower than that in enamel.[1, 2] The decreased mineral content in dentin is compensated for by an equivalently elevated weight fraction of organic constituents (i.e. 28 wt. %).[2] Although the Ca:P ratios in both calcium and dentin are about 2.1 and 2.0, respectively,[1] the specific surface areas differ markedly: 77 m²/g for enamel and 94 m²/g for dentin.[3] One reason for the increased surface area of dentin may be attributed to collagen tubules manifested within the dentin matrix, which help provide structure and support.[4] While further discussion regarding dentin and the supportive tissue in and about the tooth is quite interesting in its own right, for purposes of the present discussion, we will only highlight the enamel component, as this is the tissue that experiences the most significant mineral changes through demineralization (i.e. loss of mineral constituents) and remineralization (incorporation of new mineral constituents) events naturally transpiring in the oral environment.

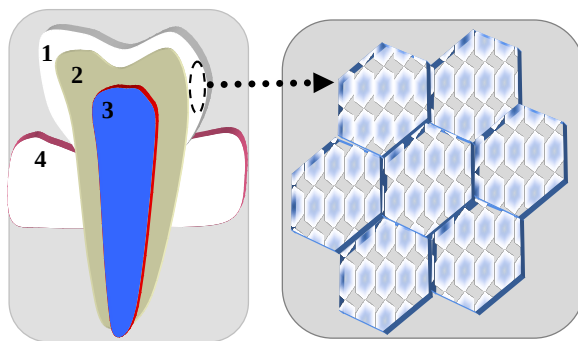
Tooth enamel is comprised of a heterogeneous arrangement of inorganic mineral and less than 1% organic substances, including proteins and collagen.[5] The overall thickness of human enamel can be up to several millimeters and visually, the apatite crystals bear a hexagonal arrangement (Schematic 1), although the actual unit cell pertaining to hydroxyapatite is likely comprised of two different sets of monoclinic crystal arrangements, with one monoclinic arrangement arising from two hexagonal cells.[6] Transmission electron microscopy experiments reveal sound enamel crystallites have dimensions of about 40 nm wide and at least 300 nm long,[5, 7] with adjacent prisms separated interproximally by a substance comprised of organic and inorganic matter that is somewhat more resistant to caries formation.[8, 9] Predominantly, the apatite crystals found in enamel manifest hydroxyl or carbonate species. Additionally, these crystals may also have magnesium, strontium, and sodium substituted for calcium.[10]

Dissolution of the enamel prisms occurs during an acid attack leading to demineralization. The nature of the acid challenge is distinguished between cariogenic and erosion; for the latter, acid reflux or consumption of acid beverages, for example, leach mineral from the crystal, creating macroscopically smooth and rounded characteristics of the enamel, while the microstructure reveals significant pitting and jaggedness.[11, 12] With respect to the cariogenic type of enamel weakening, acid-producing bacteria, such as *Streptococcus mutans*, adhere to the tooth and ferment carbohydrates containing sucrose consumed during eating events.[13, 14] This activity produces lactic acid which then eats away at the enamel structure to create subsurface lesions that may ultimately progress to cavitations, or dental caries. But where in the enamel structure does demineralization begin? Microscopically, this process occurs primarily at the center surface of the enamel prism and propagates downwards through the body of the prism, ultimately proceeding outwards to the prism walls.[9, 15] As shown in Schematic 2, the prism surface manifests species such as OH⁻ and CO₃²⁻ residing at the center of the crystal. Although this occupancy helps to stabilize the

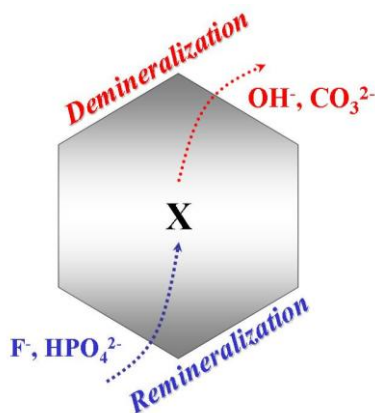
apatite structure, these species are highly prone to dissolution relative to Ca^{2+} and PO_4^{3-} , which reside on the corners and faces of the crystal arrangement.

The thermodynamic progressions of remineralization to apatite,[16] coupled with the relatively low levels of inorganic mineral constituents naturally found in saliva,[17] often are unable to keep pace with the rate of demineralization. Even more problematic, for instance, salivary flow is often reduced for individuals taking medications, including aspirin or antihistamines. It follows then the already low calcium salivary levels will likely be affected resulting in an increased risk for enamel demineralization.

Other means, such as fluoride (i.e. Schematic 2), could be required to help restore enamel strength more proactively. Fluoride has a rich and clinically-proven history of preventing dental decay.[18-21] First added to drinking water in low levels,[22, 23] topical fluorides have evolved into such common vehicles as toothpaste and mouthrinses.[24] These relatively inexpensive forms of topical dental therapy are quite appealing and will likely become even more so as the views of patients and practitioners shift even further to prevention instead of restoration, the latter of which is typically considered to be more costly and painful.



Schematic 1. Illustration of tooth cross-section and corresponding enamel microstructure. The outer mineral layer is comprised of enamel (1), dentin (2), and the pulp chamber (3). The gingival stratum encompassing the tooth is also shown (4). The enamel is comprised of hexagonally arranged calcium phosphate crystalline prisms as shown on the right.



Schematic 2. Simple depiction of remineralization and demineralization processes demonstrating the replacement of common apatite constituents OH^- or CO_3^{2-} with remineralizing agents F^- and HPO_4^{2-} agents in the core, X, at the enamel surface.

Table 1. Listing of common calcium phosphate salts and minerals used for potential hard-tissue strengthening or remineralization applications.

Common Calcium Phosphate Salts & Minerals
Calcium chloride, CaCl_2
Calcium lactate, $\text{CaC}_6\text{H}_{10}\text{O}_6$
Calcium glycerophosphate, CaGP , $\text{CaPC}_3\text{H}_9\text{O}_6$
Amorphous calcium phosphate, ACP , formula varies
Tetracalcium phosphate, TTCP , $\text{Ca}_4\text{O}(\text{PO}_4)_2$
α -tricalcium phosphate, α -TCP, $\text{Ca}_3(\text{PO}_4)_2$
Dicalcium phosphate dihydrate, DCPD , $\text{CaHPO}_4 \bullet 2\text{H}_2\text{O}$
Octacalcium phosphate, OCP , $\text{Ca}_8\text{H}_2(\text{PO}_4)_6 \bullet 5\text{H}_2\text{O}$
β -tricalcium phosphate, β -TCP, $\text{Ca}_3(\text{PO}_4)_2$
Hydroxyapatite, HAP , $\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$

Even through fluoride applications and other preventive measures, dental decay still affects the majority of the world’s population.[25] A US study conducted by the National Health and Nutrition Examination Survey recently reported that while dental decay has not increased in the last 25 years for most of the US population, dental decay is on the rise in children between 2 and 11 years old.[26] This apparent epidemic suggests that fluoride alone, despite its great clinical success and acceptance, may be insufficient.

One non-invasive and potentially high patient-compliant and acceptable approach in combating dental decay may reside in the use of calcium phosphates. Compared to calcium, saliva manifests relatively high phosphate content, and phosphate can be commonly found in acidic beverages and foodstuffs (e.g. such as dairy and vegetable products). Additionally, the public perception of calcium is very positive. Skeletal health, for instance, is maintained with sufficient calcium intake through bone modeling processes.[27] For women in particular, calcium is vital to maintaining proper bone strength, especially during pregnancy or after menopause. In terms of dental health, calcium is critically important to the preservation of the tooth structure but can easily be compromised. Therefore, it is not unusual that food and nutrition companies incorporate calcium into beverages, foodstuffs, vitamins, etc.

Thus, it is not surprising that calcium phosphate is proposed as an agent for remineralization. Promising attempts in improving dental remineralization beyond fluoride alone have included combining fluoride with calcium phosphate.[10, 28, 29] Table 1 summarizes some common calcium salts and minerals considered as candidates for enhanced enamel remineralization. These calcium forms all have varying degrees of solubility and remineralization characteristics when applied to weakened enamel. As opposed to minerals such as strontium or zinc, typically these agents are incorporated to help expedite the thermodynamic transition to apatite by elevating saturation levels of calcium and/or phosphate. Other innovative calcium phosphates that work in the absence of fluoride, such as bioactive glass, amorphous calcium phosphate, and milk-derived protein complexes containing calcium, have been developed.[30, 31] Though promising, such innovative calcium phosphates can compromise bioavailable fluoride, and therefore, therapeutic efficacy.[20] This is largely due to the reactivity of bioavailable fluoride and calcium, which rapidly form calcium fluoride[32] when introduced together in an aqueous rinse or dentifrice prior to reaching the dentition during an oral hygienic event.[33] Therefore, if one improves

fluoride efficacy through the addition of a calcium phosphate agent without compromising bioavailable calcium or fluoride, enhanced dental health benefits may be realized.

We have been reporting on studies demonstrating the synthesis and fluoride compatibility of an innovative calcium phosphate system comprised of surfactant-modified beta-tricalcium phosphate (β -TCP).[34] β -TCP is an appealing calcium phosphate system because it is a precursor to hydroxyapatite formation,[35] is biocompatible and bioactive,[36, 37] and manifests lattice defects that allow for crystal modification.[38] Sodium lauryl sulfate (SLS) is attractive due to its surface-active properties and widespread use in dental formulations.[39, 40] By using a mechanochemical process we hypothesize that a functionalized β -TCP material (fTCP) can be constructed by exploiting the defects in the TCP lattice with SLS.[41] To demonstrate the therapeutic potential of fTCP when combined with fluoride and progress towards a mechanism of action for remineralization, we evaluated remineralization efficacy as a function of fluoride and fTCP concentration. The *in vitro* efficacy studies described in this chapter subjects non-cavitated enamel specimens to remineralization and demineralization events and are based on widely-accepted *in vitro* dental models that successfully emulate the clinical situation.[20, 42-46]

2. MATERIALS AND METHODS

2.1. fTCP Preparation

The 'functionalized' β -tricalcium phosphate, fTCP, hybrid material was created using a high-impact grinding procedure described as follows. 98 wt. % β -tricalcium phosphate (EP-grade, Budenheim) and 2 wt. % sodium lauryl sulfate (NF-grade, Spectrum Chemical) were loaded into a 500 mL stainless steel grinding jar lined with yttrium-stabilized zirconium oxide. Also added to the jar were ten 20-mm diameter yttrium-stabilized zirconium oxide balls and 200 mL of pentane (> 99%, Sigma Aldrich), which served as a lubricant during grinding. A lid lined with zirconium oxide was then clamped tightly to the grinding jar and the grinding jar assembly was placed into a rotatable station mounted on the turntable of a PM400 planetary ball mill (Retsch), where it was secured with a quick-action locking spider clamp. Two identical sets of jars were secured in the PM400 to ensure necessary counterbalancing. The turntable speed was set to 375 rpm (jar speed was 750 rpm in the direction opposite the turntable rotation) and the grinding process lasted for a period of two hours, with less than 5 rpm in speed variation as monitored with a CDT-100 hand-held tachometer (Check-Line). After the grinding process was completed, the jar system was carefully opened in a fume hood with the resultant slurry deposited into a collection pan fitted with a sieve. The pan was then placed into a vacuum oven at $\sim 45^\circ\text{C}$ (Fisher Scientific) and gently evacuated to a pressure of -29 in Hg (Ashcroft) at which point the pressure inside the vacuum oven did not increase when the oven valve to the vacuum pump was closed for at least 30 seconds. The off-white dried powder was then funneled into graduated HDPE plastic bottles and stored until use. The loss on drying was near 0.0% (USP method <731>) and the true density (Micromeritics AccuPyc 1330 Helium Pycnometer) was 2.97 g/cc (USP method <699>).

2.2. Fluoride Bioavailability

The bioavailable (i.e. ionic) fluoride from each test group was determined as follows. One milliliter of the test group was added to one milliliter of TISAB II ionic strength adjuster to create a 1:1 dilution. A fluoride-sensitive electrode was then used to measure the level of ionic fluoride in millivolts. This process was repeated in triplicate, with fresh samples used for each measurement. Using standards of 10, 100, 500, 1000, and 5000 ppm fluoride (from NaF in a 1:1 ratio with TISAB II), a calibration curve was generated to convert millivolts to ppm fluoride.

2.3. Specimen Preparation and White-Spot Lesion Formation

Preparation and execution of *in vitro* performance testing on bovine enamel were performed as follows. 3 mm enamel cores were drilled from clean bovine[47] incisors and mounted in acrylic rods. The cores were then ground with 600 grit sandpaper for 2 minutes and polished with 0.05 micron gamma alumina for 45 minutes. After sonication and rinsing with distilled (DI) water, specimens were immersed in vials containing carbopol-lactic acid solution partially saturated with hydroxyapatite (BioRad) and adjusted to a pH of 5.0.[42, 48] These vials were subsequently loaded into an incubator at 37°C to establish ‘white-spot’ (non-cavitated) lesions, the characteristics and histology of which have been discussed in detail previously by White.[48] Briefly, the caries-like lesions produced by this solution have a dense surface mineral zone approximately 15 microns thick (via polarized light microscopy),[48] while lesion depth extends to about 70 µm (via microradiographic analysis[48] and reflective microscopy). After immersion for 36 hours, specimens were removed from the solution, rinsed with DI water, and measured for baseline Vickers microhardness (200 gf, 15 sec dwell time) using a microhardness indenter (Leco, model 247AT) interfaced with Confident software (Leco). Specimens exhibiting mean Vickers hardness numbers (VHN) between 25 and 45 were selected for the study and stratified into the eight groups (N=9) having an overall mean VHN of about 33 VHN (note: for reference, sound bovine enamel typically has a VHN between 300 and 350).

2.4. *In Vitro* pH Cycling Model for Remineralization Efficacy

The *in vitro* pH cycling model[49] employed to evaluate the reversal of white-spot enamel lesions throughout this paper is summarized in Table 2. Each day included two 2-minute treatment periods performed an hour apart in the morning, followed by one 4-hour carbopol-lactic acid challenge, and finally two additional 2-minute treatment periods in the afternoon. Each treatment was comprised of 5 mL test group plus 10 mL distilled water. The number and characteristics of each test group are discussed in the context of each pertinent study presented in the Results section and always involved a negative control (i.e. distilled (DI) water) and a positive control (i.e. fluoride solution). Between the daily treatments and acid challenge, specimens were immersed in artificial saliva.[43] The treatment and saliva systems were magnetically agitated at 300 rpm, while the acid challenge was static. After each treatment and acid challenge, the specimens were rinsed with DI water prior to

placement into artificial saliva. Four fresh treatment slurries and fresh carbopol-lactic acid solution were used daily, with the artificial saliva solution changed once daily after the third treatment.

2.5. Specimen Remineralization: Surface and Profile Microhardness

At the end of each pH cycling study, enamel specimens were examined for surface and longitudinal microhardness. For surface microhardness studies, the change in Vickers hardness number (ΔVHN) was determined as the difference between the post and baseline values ($\Delta\text{VHN} = \text{VHN}_{\text{post}} - \text{VHN}_{\text{base}}$), with the post value determined using the same Vickers indenter conditions (200 gf, 15 sec dwell time). Following surface analysis, samples were either examined for fluoride content by extracting enamel biopsies, or they were cross-sectioned and their longitudinal microhardness was measured.

Longitudinal assessments of the enamel cross-sections provide additional insight into bulk remineralization. A Lapcraft Lil' Trimmer circular saw was used to section the enamel specimens. The sections were then mounted with ClaroCit methylmethacrylate-based cold mounting resin (Struers) with the freshly cut surfaces exposed. The mounted specimens were serially ground with 180, 600, 1000, and 1500 grit sandpaper (3M), and then serially polished with 9, 3, and 1 μm polycrystalline diamond suspension (Buehler). Longitudinal microhardness was performed with the Knoop indenter fitted on the microhardness tester. A series of three indentations per specimen were made under 1) a 10 gf load at 12.5, 25, 37.5, and 50 μm , 2) a 25 gf load at 25, 37.5, and 50 μm , and 3) a 50 gf load used at increments between 25 and 300 μm below the specimen surface, resulting in a total of 57 indents per specimen. The resultant Knoop indentation lengths were then converted to Knoop penetration depths.

Table 2. Outline of daily remineralization and demineralization events and duration employed in the pH cycling model evaluating reversal of white-spot enamel lesions.

Event	Duration
Treatment #1*	2 minutes
Artificial Saliva, pH =7.0	1 hour
Treatment #2	2 minutes
Artificial Saliva, pH =7.0	1 hour
Acid Challenge, pH = 5.0	4 hours
Artificial Saliva,** pH =7.0	1 hour
Treatment #3	2 minutes
Artificial Saliva, pH =7.0	1 hour
Treatment #4	2 minutes
Artificial Saliva, pH =7.0	Overnight

*On Day one, specimens were pre-conditioned for one hour in artificial saliva prior to the first treatment.

**Artificial saliva was refreshed daily after the acid challenge.

Table 3. Bioavailable fluoride for NaF solutions and suspensions determined using a fluoride-sensitive electrode.

System	Mean ± SD Bioavailable F ⁻ , ppm
DI Water	< 0.4
500 ppm F	505.2 ± 0.0
500 ppm F + 80 ppm fTCP	511.0 ± 1.9
950 ppm F	946.9 ± 8.3
950 ppm F + 500 ppm fTCP	946.9 ± 8.3
5000 ppm F	4965.1 ± 11.9
5000 ppm F + 800 ppm fTCP	4944.5 ± 11.9

2.6. Fluoride Uptake: pH Cycling and Single Treatment Exposure

Depending on the dental model used, fluoride uptake measurements were performed using two different methods. For pH cycling studies such as that described above and in Table 2, enamel biopsies were microdrilled to a depth of 100 μm using a 33 1/3 inverted cone dental bur that produced hole diameters between 600 and 700 μm as measured via the Confident software. The resultant powdered enamel was then placed in a plastic dish and dissolved step-wise by adding 20 μL of 0.5 N HClO₄ followed by 80 μL of 1:1 citrate:distilled water solution. TISAB II was then added to the sample solution (1:1) and measured for fluoride content using a fluoride-sensitive electrode (Orion) calibrated to known standards and an Accumet AR Dual Channel pH meter. The measured fluoride was subsequently converted to micrograms of fluoride (μg F) per volume of drilled enamel.

Alternately, fluoride measurements after a single exposure of the white-spot enamel lesions to fluoride in a manner consistent with the Food and Drug Administration (FDA) Test Method #40 were performed as follows. Sound upper, central bovine incisors were selected and cleaned of all adhering soft tissue. A core of enamel 3mm in diameter was prepared from each tooth by cutting perpendicularly to the labial surface with a hollow-core diamond drill bit and mounted in an acrylic rod. The specimens were ground and polished to a high luster with Gamma Alumina (0.050 microns) using standard methods. The resulting specimen was a 3mm disk of enamel with all but the exposed surface covered with acrylic. An incipient white-spot' lesion was formed in each enamel specimen by immersion into a 0.1M lactic acid plus 0.2% Carbopol 907 solution for 20 hours @ 37°C. After this lesion formation step, specimens were divided into groups (N=10) and treated in a 1:3 dilution with distilled water (5 mL rinse: 15 mL DI water) under constant stirring (350 rpm) for 30 minutes. Following treatment, the specimens were rinsed with distilled water and enamel biopsies taken by drilling 100 μm deep using a microdrill bit (~ 600 μm diameter). The enamel powders were collected and dissolved in a solution comprising 1N HClO₄ and a citrate-water buffer and measured for fluoride content using a fluoride-sensitive electrode calibrated against fluoride standards (0.02, 0.04, 0.1, 0.2, 0.4, 1.0, and 2.0 ppm F).

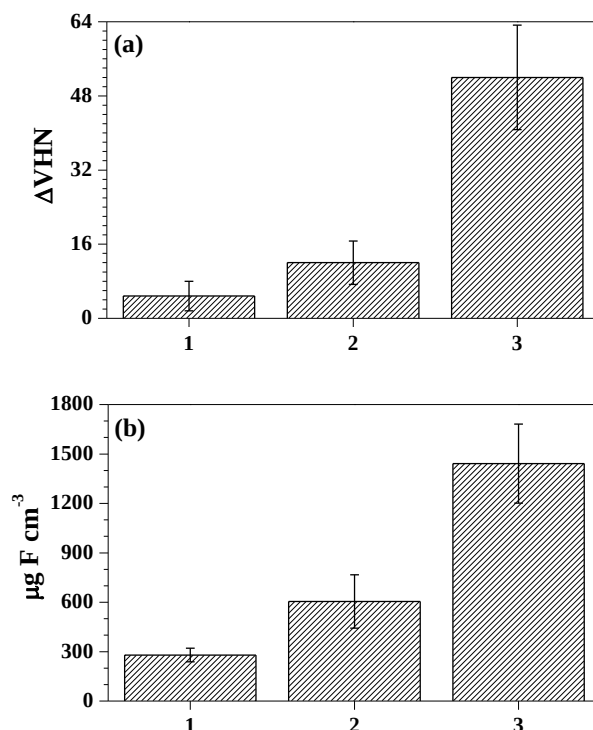


Figure 1. Mean $\pm$ SEM data are shown for (a) the change in Vickers microhardness from baseline ($\Delta VHN = VHN_{post} - VHN_{base}$) and (b) fluoride uptake into enamel specimens ($N=3$) after six days of pH cycling for Groups 1: distilled water; 2: 500 ppm F; 3: 500 ppm F + 100 ppm fTCP.

2.7. Statistics

Statistical evaluation of the microhardness data was performed using Sigma Stat software version 3.1. One-way analysis of variance (ANOVA) was used to test for significance, followed by the Student-Newman-Keuls (SNK) method to identify differences among groups ($p < 0.05$). Discordant values were excluded using the Q-test.

3. RESULTS

3.1. Fluoride Bioavailability

Bioavailable fluoride measurements in the NaF solutions and suspensions are summarized in Table 3. The distilled (DI) water had less than 1 ppm of measureable ionic fluoride. Incorporation of 80 ppm fTCP into the NaF(aq) solution produced nearly the same level of bioavailable fluoride relative to the 500 ppm F control; likewise, fTCP produced statistically similar levels of bioavailable fluoride for both 950 and 5000 ppm F.

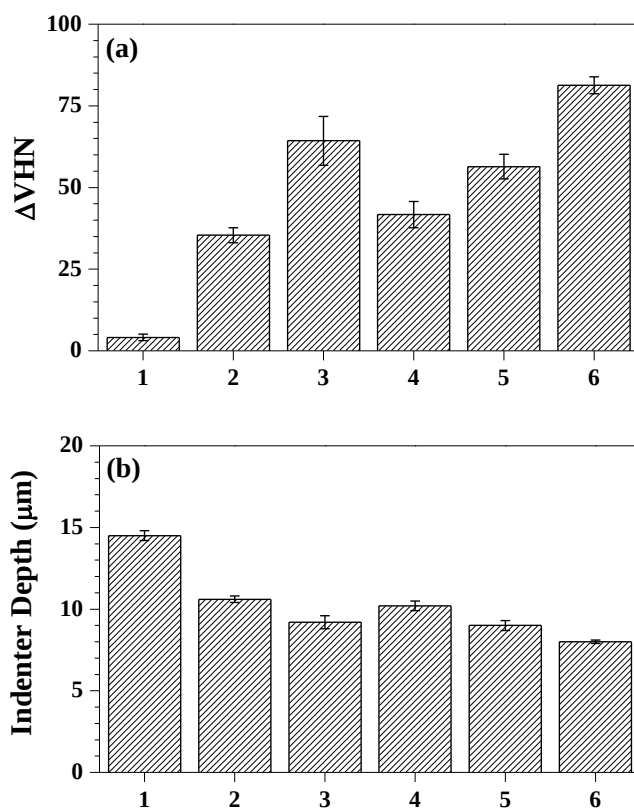


Figure 2. Mean $\pm$ SEM data are shown for (a) the change in Vickers microhardness from baseline ($\Delta VHN = VHN_{\text{post}} - VHN_{\text{base}}$) and (b) penetration depth of Vickers indenter into enamel specimens after five days of pH cycling for Groups 1: distilled water (N=9); 2: 950 ppm F (N=10); 3: 950 ppm F + 100 ppm fTCP (N=10); 4: 950 ppm F + 500 ppm fTCP (N=10); 5: 5000 ppm F (N=9); 6: 5000 ppm F + 100 ppm fTCP (N=9).

3.2. *In vitro* Remineralization via pH Cycling: 500 ppm F

The results from a six-day pH cycling study evaluating 500 ppm F with and without fTCP are shown in Figure 1. These data were generated following the protocol outlined in Table 2. In Figure 1a, the Vickers microhardness for the specimens treated with 500 ppm F is about 2.5 times greater than that of DI water. Additionally, in Figure 1b the fluoride uptake for 500 ppm F is about 4 times greater than DI water, the content of which largely reflects indigenous levels of fluoride naturally incorporated into the bovine tooth during consumption events. When fTCP is incorporated into the 500 ppm F solution, Figure 1a reveals a near quadrupling surface strengthening effect relative to the control 500 ppm F solution, while Figure 1b reveals an improvement of about twice as much fluoride uptake into the enamel relative to the positive control. The mean ($\pm$ SEM) penetration depths of the Vickers indenter on the enamel surfaces for the three groups were $12.2 \pm 0.7 \mu m$, $11.4 \pm 0.5 \mu m$, and $8.8 \pm 0.5 \mu m$ for DI water, 500 ppm F, and 500 ppm F + 100 ppm fTCP, respectively.

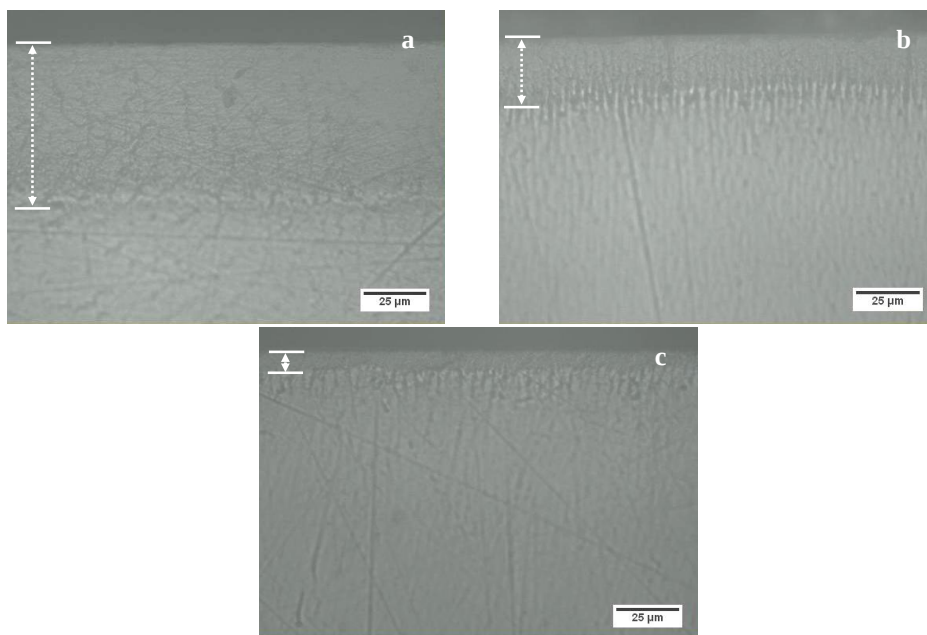


Figure 3. Optical images showing the subsurface lesion and sound enamel cross-sections after five days of cycling when treated with a: distilled (DI) water; b: 950 ppm F; and, c: 950 ppm F plus 100 ppm TCP-SLS. The bulk of the lesion area is indicated with the white bars and arrow.

3.3. *In Vitro* Remineralization via pH Cycling: 950 and 5000 ppm F

Figure 2 summarizes the results after a five-day pH cycling study (Table 2) evaluating the following groups: Group 1: DI water; Group 2: 950 ppm F; Group 3: 950 ppm F + 100 ppm fTCP; Group 4: 950 ppm F + 500 ppm fTCP; Group 5: 5000 ppm F; and, Group 6: 5000 ppm F + 100 fTCP. Statistical analyses reveal that remineralization efficacy in terms of increases in Vickers microhardness are as follows: Group 1 < Group 2 < Group 4 < Group 5 ≤ Group 3 < Group 6. Again, model validity was confirmed with the negative control statistically inferior to fluoride-containing groups. An important result here, the model also demonstrates statistically significant dose response sensitivity to fluoride with 5000 ppm F providing about 59% greater remineralization power relative to 950 ppm F. With respect to the 950 ppm F series, an improvement of 81% and 18% were obtained with 100 ppm and 500 ppm fTCP, respectively. 100 ppm fTCP plus 5000 ppm F resulted in a 44% increase in microhardness relative to fluoride alone. Additionally, the penetration depths of the Vickers indenter in Figure 2b are listed in decreasing depths as follows: Group 1 < Group 2 ≤ Group 4 < Group 3 ≤ Group 5 < Group 6. The deepest penetration occurred for the DI water group, with the 5000 ppm F groups providing the least penetration. Both 950 ppm F and 950 ppm F plus 500 ppm fTCP yielded similar penetration depth resistance, while 950 ppm F plus 100 fTCP provided almost the same resistance relative to 5000 ppm F. Representative optical images depicting the nature of the white-spot lesion when treated with DI water, 950 ppm F, and 950 ppm F plus 100 fTCP are shown in Figure 3. The size of the subsurface lesion clearly diminishes when treated with fluoride and fluoride plus fTCP. To probe the extent of bulk remineralization, specimens from the following groups were cross-sectioned and analyzed

using the Knoop's indenter: DI water, 950 ppm F, 950 ppm F plus 100 fTCP, and 950 ppm F plus 500 ppm fTCP. Trends revealing the depth of the Knoop's indenter are presented in Figure 4 in three panels, with data generated under a 10 gF load for (a), 25 gF load for (b), and a 50 gF load for (c). The sensitivity of the Knoop's indenter enables one to gauge the sensitive remineralized regions at various depths within the subsurface lesion. For all three panels, DI water yielded the deepest penetration of the Knoop's indenter. Under a 10 gF loading level, measurements were made at 12.5, 25, 37.5, and 50 μm . Statistically, the 950 ppm F plus 100 ppm fTCP provided the greatest resistance to indenter penetration at 12.5 and 25 μm , beyond which all three 950 ppm F groups converged to yield equivalent resistance. In panel 4b, a 25 gF load demonstrated that both fTCP levels statistically outperformed the 950 ppm F group at both 25 and 37.5 μm . This also followed with a clear distinction in the slopes with the steepness modified significantly for the fTCP groups. Finally, the 50 gF trends in panel 4c demonstrate differences only at the 50 μm ; at this depth, both fTCP groups provide significantly better resistance to indenter penetration relative to fluoride alone. Beyond this depth sound enamel lying beneath the subsurface lesion is ultimately reached as evidenced by the similarities among the four groups.

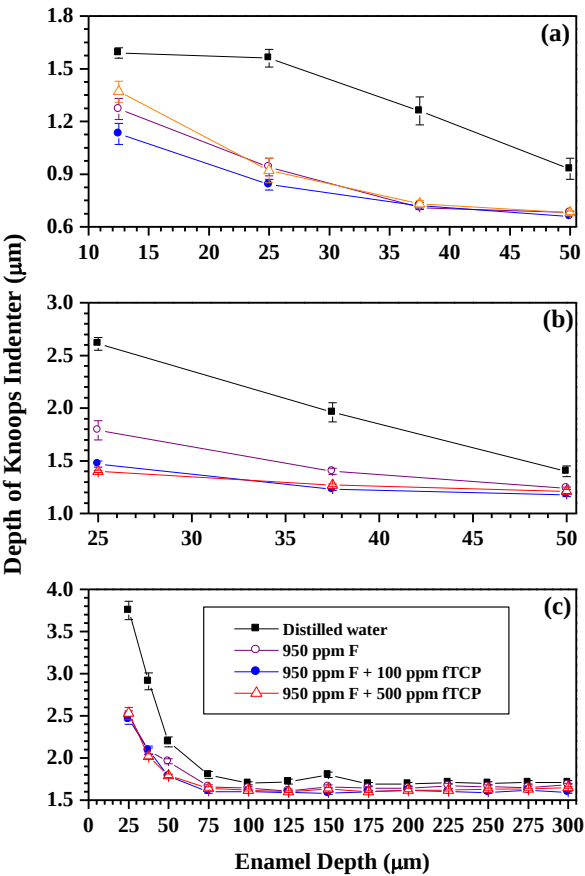


Figure 4. Knoop's indenter penetration depth (error bars represent the standard error of the mean) along the body of subsurface enamel cross-sectioned lesions after five days of cycling. Knoop's indenter measurements were made under a 10 gF load (a), 25 gF load (b), and 50 gF load (c). The legend in (c) also refers to both (a) and (b). Enamel depth refers to distance from the outer enamel surface.

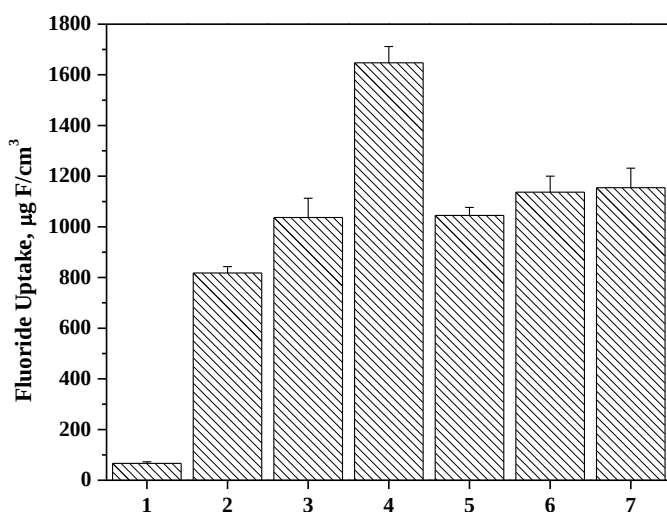


Figure 5. Mean fluoride uptake ($N=12$, $\mu\text{g F/cm}^3 \pm \text{SEM}$) into a single 30-minute exposure of white-spot enamel lesions to Groups 1: distilled water; 2: 500 ppm F; 3: 950 ppm F; 4: 5000 ppm F; 5: 500 ppm F + 80 ppm fTCP; 6: 950 ppm F + 500 ppm fTCP; 7: 5000 ppm F + 800 ppm fTCP.

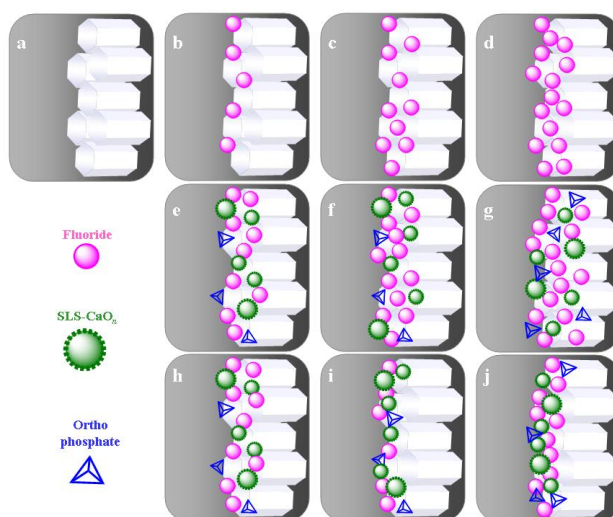


Figure 6. Cartoons describing remineralization phenomena occurring when white-spot enamel lesions (a) are treated with 500 ppm F (b), 950 ppm F (c), 5000 ppm F (d) solutions in either a pH cycling model or single-treatment fluoride uptake study. These cartoons contrast to lesions treated with fTCP plus 500 ppm F (e,h), 950 ppm F (f,i), and 5000 ppm F (g,j) with the second and third rows of images pertaining to results from a pH cycling model and a single-treatment fluoride uptake study, respectively.

3.4. *In Vitro* Fluoride Uptake: 500, 950 and 5000 ppm F

The results from a single 30-minute treatment on white-spot enamel lesions with the following aqueous groups with and without NaF and fTCP: 1: DI water; 2: 500 ppm F; 3: 950

ppm F, 4: 5000 ppm F; 5: 500 ppm F + 80 ppm fTCP; 6: 950 ppm F + 500 ppm fTCP; and 7: 5000 ppm F + 800 ppm fTCP. The mean fluoride uptake results are presented in Figure 5 with Group 1 < Group 2 < Group 3 = Group 5 < Group 6 = Group 7 < Group 4. The model demonstrated a fluoride dose response effect and the fluoride taken into the specimens treated with DI water represents indigenous levels. There was a 28% increase in fluoride uptake for 500 ppm F plus 80 ppm fTCP relative to 500 ppm F. The fluoride uptake for 950 ppm F plus 500 ppm fTCP was equivalent to the uptake generated from 950 ppm F. There was a significant decrease in fluoride uptake for 5000 ppm F plus 800 ppm fTCP relative to 5000 ppm F.

4. DISCUSSION

Bioavailable fluoride does not appear to be compromised with the addition of fTCP. This simple assessment is actually quite critical to the potential success of any combination of bioavailable fluoride and bioavailable calcium. Typically, bioavailable calcium and fluoride react with one another to produce CaF_2 prior to contact with the dentition. As a result, attempts to combine the two agents must rely on either anhydrous formulations or split-compartment designs;[50, 51] otherwise, bioavailable fluoride would be affected, thus directly compromising therapeutic efficacy.[52] This unwanted result typically limits most innovative forms of calcium phosphate currently proposed for possible introduction into dental vehicles containing fluoride (i.e. toothpaste, mouthrinse, etc).[52] Hence, one benefit of the fTCP technology is that it does not compromise fluoride bioavailability. The data collected here were taken after a couple of days, however, we have reported on fluoride availability under accelerated aging conditions, whereby fluoride compatibility was preserved.[34] To date, this is the first form of bioavailable calcium that is compatible with NaF in both an aqueous and single-compartment format.

The pH cycling model (Table 2) employed in these studies accurately emulates clinical results for early caries reversal.[20] The alternating sequence of saliva-treatment-saliva-acid challenge-saliva-etc is designed to mimic crystal seeding (i.e. treatment) and growth (i.e. saliva) events, as well as enamel weakening, or demineralization, events (i.e. acid challenge) that naturally occur during eating events. Physiologically, the pH of the oral cavity drops to below 5.5 (the approximate solubility of tooth enamel) leaving the enamel susceptible to ‘white-spot’, or subsurface lesion, formation, where the acids diffuse through the plaque fluid and pellicle layers to interface with and dissolve apatite (i.e. demineralization).[13, 53] These white-spots indicate the onset of caries and if not repaired (i.e. remineralized) ultimately progress to cavitations. The lesions are characterized by a mineral rich surface layer, which forms due to the pellicle and plaque fluid layers that inherently reside on the tooth surface to prevent ‘free-flow’ mineral loss from the tooth. Directly underneath the mineral-rich layer is the mineral-depleted layer, which extends to meet sound enamel with a bowl-like concavity.[54]

The statistical differences realized in Figure 1 demonstrate the model can distinguish between negative and positive controls, even with a modest number of specimens (N=3) per group. Furthermore, the model is also appropriate for evaluating potentially synergistic effects when fTCP is combined with fluoride. These pilot results clearly demonstrate a

synergy between fTCP and fluoride, with remineralization extending to about 8.8 μm below the enamel surface compared to 11.4 μm 500 ppm F alone. These results may suggest that improved dental benefits can be gained if a lower level of fluoride is used in conjunction with fTCP. Although these results bear great promise for low levels of fluoride (i.e. mouthrinses, children's toothpaste, etc), what about higher levels of fluoride, such as those found in over-the-counter (OTC) (i.e. between 950 and 1150 ppm F) and professional (i.e. 5000 ppm F) dentifrices? A closer look into the relationship at higher fluoride and fTCP concentrations is examined below.

Figure 2 reveals a significant look into the differences among fluoride-containing groups and fTCP. While 100 ppm fTCP improved the efficacy of 950 ppm F by 81%, 500 ppm fTCP drops the efficacy level down to about 18%. Based on this study, apparently a five-fold increase in fTCP somehow inhibits remineralization near the enamel surface. At these two concentrations, the Vickers indenter penetrates to mean depths of 9.2 μm and 10.2 μm , respectively, with the latter comparing very similarly to the mean depth of 10.6 μm for 950 ppm F. In addition to a level of 100 ppm fTCP significantly improving 500 ppm F efficacy (Figure 1), 5000 ppm F efficacy is also improved by 44%. Thus, we have two scenarios to explore: 1) the net remineralization benefit arising from fixed fTCP content and varied fluoride concentration and 2) that arising from fixed fluoride concentration and varied fTCP content.

For the first scenario at 100 ppm fTCP, there is a decline in the ability of fTCP to provide net surface remineralization improvements with increasing fluoride content from 500 ppm F to 5000 ppm F. This is not the limitation of the fTCP, however, as much as it is the properties of fluoride; hence, this suggests the reaction kinetics of fluoride become modulated at elevated fluoride levels with the limiting remineralization step shifting towards thermodynamic control. Apparently, fTCP interacts with the tissue or helps to retain bioavailable fluoride near the surface of the tooth. The optical images presented in Figure 3 demonstrate fTCP helps to build mineral by reducing the size and porosity of the white-spot enamel lesion. Still, a single-treatment fluoride uptake study to be discussed later in this chapter seems to indicate that fTCP serves a dual role depending on the level of bioavailable fluoride. However, at elevated fTCP levels (i.e. 500 ppm fTCP) at a fixed level of fluoride (i.e. 950 ppm F) it is clear that fTCP may partially compete for bioavailable fluoride with the enamel surface; hence, bioavailable fluoride ions and fTCP not jointly participating in mineral integration at the enamel surface will likely interact with each other.

But what about the nature of remineralization occurring deeper within the enamel lesion? The second scenario can be further explored by examining the trends in microhardness along the enamel lesion cross-section as shown in the three panels in Figure 4. The data collected near the enamel surface at 12.5 μm in Figure 4a mimic the surface microhardness results to provide additional support for the extent and nature of the remineralization occurring from each group. We note the 25 and 50 gF loading levels were excessive at this delicate enamel depth; hence, a 10 gF load was used. At depths between 25 and 37.5 μm , either the sensitivity of the 10 gF load is unable to discern differences among the fluoride groups or remineralization has reached an equilibrium among the groups. Using a 25 gF load, the cross-sections were also examined as shown in Figure 4b. Notably, the slopes of the data trends appear to be distinguished between fluoride and fluoride plus fTCP, with the latter having a shallower slope. Importantly, there appears to be remineralization persisting up through 37.5 μm . The obvious slope differences between DI water and fluoride clearly indicate the nature

of remineralization is unique to the presence (or absence) of fluoride, as shown in the optical images in Figure 3. The indents generated under 10 gF and 25 gF loads suggest non-uniform remineralization throughout the body of the lesion. Such non-uniformity may result from differences between the groups and the nature of 'new' mineralized layers. Distinctions, however, begin to diminish among the three fluoride groups between 37.5 and 50 μm , indicating that once again either the load is insensitive to subtle remineralization differences or that remineralization is nearing a limit. Figure 4c clearly shows the 50 gF load is too heavy and fails to distinguish differences among the fluoride groups otherwise observed when probed with the 10 and 25 gF loads, optical microscopy, and surface microhardness. Hence, any potential differences among the fluoride groups are neutralized. However, differences are discernable at the 50 μm depth, indicating the slopes of remineralization are unique for fluoride and fluoride plus fTCP. These differences suggest the presence of fTCP effectively alters the 'typical' bulk remineralization processes that inherently develop with just fluoride. We note that the original subsurface lesion persisted down to a depth of 70 μm from the enamel surface,[48] so distinction at the 50 μm mark, which is nearing the border of the lesion, appears reasonable. At depths of 75 μm and beyond, sound enamel is ultimately reached.

Consideration of these data suggests that fTCP contributes significantly to remineralization. Furthermore, the non-linear tissue response with increasing fTCP loading marks a distinction from the observed fluoride dose response effect observed in Figure 2. Hence, in the presence of fluoride, one cannot expect to simply increase fTCP, as one does with fluoride, in order to effectuate substantial subsurface enamel remineralization (both surface and bulk). The efficacy gained when fTCP is added to fluoride appears to act through a synergistic effect, where the ultimate benefit is not simply a sum of the individual remineralization benefits of the fTCP and fluoride constituents. Due to the reaction kinetics of fluoride, increasing the flux of fluoride to 5000 ppm F, for example, will result in extensive surface remineralization compared to 950 or 500 ppm F, but this does not translate into bulk remineralization.[55] The incorporation of fTCP, on the other hand, appears to extend remineralization benefits deeper within the subsurface region. To gain further insight into the synergistic interactions between fluoride and fTCP, a single-treatment study was performed and the results are discussed below.

The results summarized in Figure 5 clearly indicate white-spot enamel lesions are sensitive to the level of fluoride. Additionally, it is also clear that fluoride uptake is favored at low fluoride, low fTCP concentrations. Fluoride uptake is neutralized, however, at the 950 ppm F mark when 500 ppm fTCP is added. Doubling the fluoride level to 950 ppm F combined with quintupling the fTCP level to 500 ppm fTCP clearly does not produce enhanced fluoride uptake. Finally, quintupling the fluoride level to 5000 ppm F and adding 800 ppm fTCP significantly impairs fluoride uptake relative to the control. While the 500 ppm F data appear consistent with pH cycling data shown in Figure 1, the data for 950 ppm F and 5000 ppm F are not, despite the fact fTCP does not compromise fluoride bioavailability (Table 3). These surprising results offer an opportunity to further glean information regarding the interactions among fTCP, fluoride, and the enamel. Since fluoride bioavailability is not compromised, fluoride and fTCP must be interacting with the enamel surface regardless of fluoride level. pH cycling data clearly demonstrates fTCP is able to aid remineralization throughout the bulk of the enamel lesion. At 500 ppm F, the level of fluoride is low enough to permit constructive permeation with the dental tissue to presumably encourage both fTCP and

fluoride uptake. fTCP will naturally adhere to the enamel surface due to each of the two components comprising the fTCP: tricalcium phosphate and sodium lauryl sulfate. Interestingly, sodium lauryl sulfate has repeatedly been shown to inhibit remineralization and fluoride uptake.[39] In our studies, impairment only occurs during the single-treatment study for high fluoride levels. The competition for available reaction sites at the enamel surface is likely the limiting factor affecting fluoride penetration in the presence of fTCP at mid-to-high levels of fluoride. fTCP was engineered as a surface-active species that would help facilitate heterogeneous mineral nucleation in weakened enamel.[34, 56, 57] Based on pH cycling data, higher levels of fTCP at 950 ppm F may help bulk remineralization, but do not enhance surface remineralization.

In the low-fluoride, low-fTCP limit, reaction at the enamel surface is not compromised presumably due to the relatively high number of nucleation sites manifested in the subsurface lesion relative to fluoride and fTCP. With moderate levels of fluoride (i.e. 950 ppm F), however, both remineralization and fluoride uptake suffer at 500 ppm fTCP; these data suggest that at sufficiently high fTCP concentrations, fTCP may behave similarly to pyrophosphates or biphosphonates.[58, 59]. Another possibility is that fTCP integrates into the enamel surface. Our previous studies have shown that increased microhardness can be obtained with 1100 ppm F plus 500 ppm fTCP even though fluoride uptake is *not* improved.[34] Therefore, fTCP contributes fundamentally to the mineral seeding and subsequent mineral growth within the subsurface lesion. Interestingly, remineralization with 5000 ppm F plus either 100 ppm fTCP or 800 ppm fTCP[41] is not inhibited, although the fluoride uptake data in Figure 5 clearly reveals impairment. Because remineralization extending to the bulk persists, we presume fTCP permeates throughout the porous lesion, where a dynamic environment is created due to alternating immersion in acidic and neutral pH environments. Thus, such permeation is made possible in a pH cycling study as opposed to a single treatment experiment. Since calcium naturally responds to weakened enamel and both orthophosphate and the sulfate head group bear favorable permeability properties,[60] it is highly likely these constituents interface with enamel surface competitively with fluoride; relatively speaking, the fluoride ion manifests weaker permeability relative to PO_4^{3-} and SO_4^{2-} . In the limit of high fluoride content (i.e. 5000 ppm F), fTCP (e.g. 100 ppm or 500 ppm fTCP) effectively reacts at the surface and reduces fluoride penetration; as a result, fluoride may interact with fTCP to create metastable fTCP-fluoride clusters near the surface of enamel. To help visualize these various limits, a schematic is provided in Figure 6 demonstrating subsurface enamel lesion response to fluoride and fTCP when examined in a multiple-step (i.e. Table 2) or single-step regimen. The top row in Figure 6 demonstrates the effect of increasing fluoride from 0 to 5000 ppm F, irrespective of whether the multiple-step or single-step study is used. The second row demonstrates the response of the enamel when subjected to a pH cycling regimen. Clearly, reaction at the surface and penetration deep within the body of the lesion occur as shown through Figures 1 through 4. Finally, if a single-step procedure is employed, the penetration of fluoride is ultimately limited at high fluoride, high fTCP content as shown in the third row of Figure 6. This viewpoint may be consistent with theoretical modeling that predicts large calcium-fluoride clusters form, consisting of many ions that eventually serve to nucleate CaF_2 mineral.[32] Since this is kinetically favorable, the thermodynamics will also favor CaF_2 formation in the high fluoride limit if fluoride is unable to react sufficiently with the enamel surface.

5. CONCLUSION

Fluoride has a rich and proven history of reversing dental caries. In an attempt to further the anticaries benefits afforded by fluoride, there is a need to develop innovative biomaterials that when combined with fluoride boost remineralization efficacy without compromising fluoride bioavailability. Our approach has been to create a unique functionalized tricalcium phosphate (fTCP) using a solid-state mechanochemical process. Then, using several established dental models, we evaluated its fluoride compatibility and remineralization efficacy. fTCP was found to be stable in aqueous solutions of 500, 950, and 5000 ppm F, and is the first form of bioavailable calcium that is stable with fluoride in an aqueous and single-compartment environment. Using an *in vitro* pH cycling regimen emulating actual events transpiring in the oral cavity, we demonstrated that the baseline remineralization of softened enamel with topical solutions of 500, 950, and 5000 ppm F can be substantially improved when combined with fTCP. Using both Vickers and Knoop microhardness measurements, the reversal of white-spot lesions was attributed to both surface and bulk remineralization effects with fTCP providing superior remineralization over the base fluoride solution. However, in a single-step dental model, fTCP was found to limit fluoride uptake. This result was inconsistent with multiple-step regimens and our previously reported work. Because fTCP does not compromise fluoride bioavailability and is not critically needed to boost remineralization through enhancing fluoride uptake, we suggest that fTCP also interacts with the enamel tissue and is not solely acting as a carrier of supplemental fluoride. Hence, single-treatment fluoride uptake studies, such as that outlined in the FDA monograph for fluoride-containing dentifrices, may not be indicative of the power of fTCP. As such, further evaluation of fluoride-fTCP solutions and other formats probing fluoride's activity in dental tissue should be viewed with caution. With properly modeled regimens and studies, quantitative assessments of remineralization using microhardness and optical microscopy are valid characterization tools for probing efficacy related to the combination of fluoride and fTCP. The synergistic blend of fTCP and fluoride demonstrated in this chapter will likely bear on the future development of dental preventive products aimed at attenuating and/or reversing dental caries.

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Chapter 8

TRANSLATION OF EMERGING HYDROGEL THERAPIES: THE ROLE OF METROLOGY

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ABSTRACT

Traditionally hydrogels have been widely used in medicine for sight correction, as coupling agents, barrier coatings and vehicles for drug delivery. Such materials are relatively simple in terms of their design and performance. This situation is rapidly changing as hydrogels begin to find a use in emerging healthcare solutions particularly in regenerative medicine and advanced drug delivery. Hydrogels capable of stimuli responsiveness, self-assembly, and molecular selectivity can now be produced for therapeutic applications owing to advances in hydrogel synthesis.

Despite the growth of potential hydrogel based therapies, the route to market for new products is constrained by the need to develop an appropriate regulatory and metrological framework under which they will be assessed. This chapter will outline the role that metrology has to play in the regulation of emerging hydrogel-based therapies and detail possible strategies for assessing their safety and effectiveness. It will be concluded that there is a need to improve current metrology in relation to hydrogels in order to expedite their translation into clinical practice.

INTRODUCTION

Hydrogels are water-swollen polymeric networks, that generally contain 60% to 99% water, yet maintain the structural integrity of a solid due to the presence of cross-links [1]. This high water content coupled with their soft, rubbery nature, typically high permeability and biocompatibility makes hydrogels attractive biomaterials [2]. Traditionally hydrogels in

medicine have been used for sight correction, as coupling agents, barrier coatings and vehicles for drug delivery. Manufacturing contact lenses from very high water content, oxygen permeable hydrogels has transformed treatment strategies for sight correction. The combination of soft mechanical properties, inertness to many physiological processes, favourable optical properties and permeability to oxygen and metabolites has enabled advances in contact lens design [3] including disposable lenses, extended wear lenses, tinted lenses and bifocal lenses. The ability to design hydrogels with mechanical and electrical properties similar to soft tissue is exploited in their use as coupling agents. For example, hydrogels are extensively used as ultrasound transmission agents as their acoustic properties are well matched to that of soft tissue [3]. As barrier agents, hydrogels have been widely used to treat wounds as they have the ability to assist in wound debridement, provide protection from infection, allow oxygen access to the wound and in general accelerate wound healing [4]. Hydrogels also have application in controlled drug delivery via the use of transdermal patches [3].

The hydrogels used in these applications are relatively simple in terms of their design and performance. This situation, however, is rapidly changing as hydrogels begin to find use in emerging healthcare solutions particularly in regenerative medicine and advanced drug delivery, owing to improvements in hydrogel synthesis. Hydrogels can now be incorporated into the body either by implantation of a matrix which is in a gel state [5-10], or injection of a liquid which, through application of stimuli (e.g. temperature, pH), will gel once in the body [7, 9-15]. Injectable hydrogels have the advantage of being less invasive than implantation and are ideally suited to treat irregularly shaped defects. Thus, they can be injected into wound sites together with cells and growth factors to form self-assembled structures for use in regenerative medicine (e.g. in angiogenesis [13], re-building of nerves [16] and cartilage repair [17]). Additionally, hydrogels can also be produced that exhibit bio-mimetic behaviour that respond to enzymatic action or to the presence of specific biomolecules (e.g. antigens, oligonucleotides) [18-20]. Such materials can modulate the cellular microenvironment by presenting both biophysical and biochemical signals to regulate gene expression and ultimately cell phenotype [21].

Despite the potential market size of hydrogel based therapies, the route to market for new products is constrained by the lack of clarity in the regulatory framework under which they will be assessed as well as market acceptance and the availability of standards that can be used to ensure product consistency. The latter is particularly important for bolstering the confidence of the consumer as well as offering a degree of protection for the material producer in the case of product failure. Although progress is being made towards establishing appropriate guidelines for assessing product safety and effectiveness, there is still a need to develop guidance for clinical studies and test methods for evaluation of novel therapies. Moreover, there is a particular need for assessment strategies that provide scope for future product improvement. Further challenges lie in design of product specification and efficient manufacturing processes as well as in the implementation of process validation.

This chapter will provide insight into emerging hydrogel therapies and describe the role that metrology has to play in product regulation and detail possible strategies for assessing their safety and effectiveness. Further, the need for establishing procedural standards for hydrogel characterisation will be discussed as well as the research requirements for new and/or improved characterisation techniques.

HYDROGEL FUNCTION

There are numerous hydrogel systems available, that differ in terms of the type and permanence of the cross-link and whether they are sourced from natural or synthetic products, which exhibit a wide range of properties. However, for application in medicine, hydrogels need to be biocompatible meaning the material must not elicit an inflammatory response nor demonstrate immunogenicity or cytotoxicity [14]. They should have mechanical properties compatible with those of the local environment, sufficient permeability to promote cell and nutrient transport and degrade at the required rate to either match tissue regeneration for regenerative therapies or to release bioactive agents at an appropriate rate for advanced drug delivery [22, 23]. The setting time is also a practical consideration that must be taken into account, with some advanced therapies requiring setting within a few minutes to ensure spatial localisation of the hydrogel or to ensure that cells are encapsulated. Further, for completeness, ease of handling must be considered as it is of utmost importance for clinical use [14]. Recently, hydrogels have been developed that respond to external stimuli, self-assemble or recognise specific molecules, which are described below:

Stimuli Sensitive Hydrogels

Stimuli sensitive hydrogels undergo significant transformations (e.g. change in shape, surface characteristics, solubility, swelling capability, phase from sol-to-gel) in response to changes in their environment [24, 25]. Stimuli capable of eliciting such changes include pH, temperature, enzymatic action and solvent composition [26-34]. These systems have applicability in drug delivery for targeted, controlled release of therapeutic agents, particularly for delivery of proteins and peptides which, in the past have been challenging due to their poor chemical stability in aqueous solutions and their susceptibility to in vivo degradation. There are also applications in regenerative medicine for stimuli sensitive hydrogels, i.e. as cell delivery vehicles and injectable scaffolds. Here, their use is growing as they are less invasive than implantation and are ideally suited to treat irregularly shaped defects.

To date, most work has been carried out in the development of temperature and pH responsive hydrogels, both of which can be caused by a number of mechanisms. Here, physiological changes in temperature and pH act as triggers to agent release, the oscillation of which results in a pulsatile release profile [35]. The majority of temperature responsive systems are based on gel collapse at the lower critical solution temperature (LCST) of the polymer network [27, 28, 32, 36]. The LCST is the temperature below which the two phases of the aqueous polymer solution are miscible and above which they are immiscible forming the hydrogel [37]. This effect is due to a transition from a hydrophilic to hydrophobic structure. It has been suggested that the *rate* of this transition is dependent on the hydrophilic nature of the polymer network [36].

Work has been carried out to tailor the LCST of hydrogels to match body temperature (37 °C) using aqueous solutions of polymers that are biocompatible and biodegradable. These materials can be used as injectable tissue scaffolds and cell delivery vehicles in regenerative medicine and as vehicles for targeted drug delivery. For the latter application gelation can be

triggered by a change in the local temperature as commonly found in wound beds [31]. The LCST has been shown to depend on the composition and conformation of the polymers that form the hydrogel network [27, 30]. With this insight, hydrogels have been designed to have specific LCSTs or multiple LCSTs based on modification of the hydrophilic/hydrophobic content of the polymer network and the use of interpenetrating networks composed of different polymers. For example, co-polymerisation with hydrophobic co-monomers can result in an increase in the LCST. Multiple transition temperatures can be obtained by using multiple polymer networks with different co-monomers grafted to the polymer backbone [29, 30].

Work has also been carried out to develop pH sensitive hydrogels [31, 33, 34] which have applicability for controlled release of therapeutic agents through the gastrointestinal tract, along which significant changes in pH occur [31]. These pH sensitive systems may also be used for targeted drug treatments of cancer as a drop in pH has been observed in tumour tissue as compared to normal tissue [31]. In practice, pH sensitive hydrogels can be produced by adding pendent acidic or basic functional groups to the polymer backbone which will either accept or release protons in response to pH changes [34]. The degree of ionisation of these hydrogels depends on the number of ionisable groups in the hydrogel, from which the electrostatic repulsion originates. For example, a pH sensitive dextran-based hydrogel has been produced by cross-linking carboxylic and hydroxyl groups such that there was an excess of carboxylic groups in the system [34]. It is also possible to incorporate hydrophobic moieties into pH sensitive systems. In this case the pH at which the conformational change occurs can be modulated as the more hydrophobic the moiety is, the more repulsive electrostatic force is needed to induce a conformational change [29, 30]. Materials under development include hydrogels that are responsive to both temperature and pH, e.g. a combination of methacrylic acid and N-isopropylacrylamide (NIPAAm) [38].

Self-Assembling Hydrogels

Some classes of hydrogels have the capability to *spontaneously* self-assemble from disordered components into a higher ordered structure without guidance from an external source such as a template. Self-assembly of such systems results in a more thermodynamically stable system through a process driven by a reduction in the Gibbs free energy [39] as a result, the structures tend to be relatively free of defects. Further they can form complex architectures to promote cell attachment and proliferation [40] through careful selection of the chemical building blocks. Self-assembling gels can also be used as matrices to maintain the function of differentiated cells [41] and to promote the differentiation of progenitor cells [42] without the use of any specific biofunctional ligands [21]. In practice, self-assembling hydrogels have great potential for use in regenerative medicine as they can be injected into the body as a liquid and assemble into a gel *in vivo* providing an ideal environment for cell and tissue growth.

Although there are a number of routes to self-assembly most involve the combination of shape-complementary structures, e.g. amphiphilic peptides have been used to form bundles of thin coiled-coil fibrils that assembled into a fibrous hydrogel network [43]. Further, it has been shown that by altering the structure of the starting peptides that hydrogels composed of fibres with different thicknesses and density can be formed [24]. Particular success has been

found with self-assembling hydrogels composed of block and graft copolymers where the intermolecular interactions driving self-assembly are primarily hydrophilic-hydrophobic interactions and hydrogen bonding. In these systems coiled-coil domains have been utilised as recognition motifs resulting in the formation of precisely defined three-dimensional (3D) structures. In particular, experimental results have shown the importance of coiled-coil sequences in the self-assembly of triblock copolymers from two coiled-coil blocks flanking a random coil [1]. Further experimental work demonstrating the utility of peptides in the design of self-assembling hydrogels has been carried out using modulus peptides [25]. Modulus peptides have two distinct sides with respect to the polymer backbone; one side is hydrophilic and the other is hydrophobic which facilitates self-assembly into a structure that closely mimics the structure of the extracellular matrix [25]. As a result, these self-assembling peptides have attracted significant interest for 3D cell culture, the structure itself has been shown to facilitate cell differentiation [25]. It is likely that the complexity and control of the architecture of self-assembling hydrogels will improve with the advent of molecular engineering, resulting in superior matrices for cell culture in regenerative medicine and to tolerate high loading densities of drugs, cells, enzymes or antibodies in a very small volume.

Molecule Selective Hydrogels

Distinct from stimuli sensitive hydrogels which respond to non-specific changes in environmental parameters, molecule selective hydrogels undergo changes in response to recognition of specific molecules [19, 44-46]. Hydrogels with improved mechanical stability and biocompatibility can be produced by tailoring the structure of the shape-complementary building blocks of self-assembling systems [47]. In drug delivery these materials can be used to optimise drug affinity and drug release profiles opening up new pathways for delivery of molecular therapies [47].

Molecule selective hydrogels are based on the formation of non-covalent interactions between chemical groups in the hydrogel and specific features of the stimulant molecules. This is achieved by incorporating recognition sites into the polymers that form the hydrogel. For example, block copolymer based DNA molecules can be hybridised which enables the structure of the hydrogel to be modulated from spherical micelles to rod-like structures by introducing a long complementary strand of DNA [48]. Another approach to the design of molecule selective hydrogels involves network formation between antigen and antibody moieties grafted onto the polymer chains. Hydrogels can also be prepared by using antigen-antibody bonds at cross-linking points enabling the hydrogel structure to be selectively modulated through the addition of free antigens in solution [46], e.g. rabbit immunoglobulin G (IgG) has been used as an antigen and chemically coupled with *N*-succinimidylacrylate (NSA) to form a vinyl-rabbit IgG. The vinyl-rabbit IgG was then mixed with goat anti-rabbit IgG to form an antigen-antibody complex. Finally, the antigen-antibody bond site was entrapped in a hydrogel by copolymerisation of the complex with acrylamide and *N*, *N*'-methylenebisacrylamide [46]. A further design approach is the use of molecular imprinting which allows the formation of specific recognition and catalytic sites in polymers through the use of templates [19, 46]. The templates are used to produce cavities on the hydrogel polymer backbone of complementary size, shape and spatial distribution as the specific functional groups. Modulation of the template size will alter the mesh size of the hydrogel polymer

network enabling structures with different mechanical strength and permeability to be produced [47]. Work is also underway to develop nano- or micro-patterned materials for selective protein adsorption, cell adhesion and bioactive substance immobilisation which could be achieved by forming hydrogels on surfaces in specific micro-patterns [44, 45].

Therapy Translation

Whilst there are significant opportunities for commercialisation of new hydrogel technology, the route to market is challenging, as it is for any product used in medicine. Bearing in mind that the penultimate user of these materials is likely to be a surgeon, it is worth ensuring that the product envisaged is robust enough to be handled in the operating theatre or wherever it is to be used. Short shelf life, delicate preparative methods, susceptibility to high shear stress, as may be encountered by injectable scaffolds, can all be substantial obstacles to commercialisation. It is always worth discussing potential products with the medical profession even at the pre-development stage to ensure that they are comfortable with using such a product, especially if a viable treatment already exists (noting that the medical profession will be the product consumers). It is of course difficult to generalise about markets because the applications can, and are likely to be very diverse. However, involvement of the patients at an early stage in product development can also be advantageous e.g. religious beliefs and personal prejudices or dislikes can mean that the patient will reject a product regardless of its suitability.

Commercialisation of a new technology can also be severely handicapped by the requirement to comply with a regulatory framework that needs to be created or adapted from current regulations. This issue has been encountered in assessment of hydrogel therapies that combine drugs, devices and/or biological products. After more than a decade this issue is beginning to be resolved, as progress is being made towards the establishment of appropriate guidelines for assessment of safety and effectiveness of *combination products*. For example, the United States Food and Drug Administration (FDA) have developed guidelines for the assessment of combination products which are based on identification of the product's primary mode of action, major components and intended use of the product. This information is then used to determine the set of regulations the product should comply with, further details can be found in documentation on the FDA website [49]. This activity, by a regulatory body, ensures a 'relatively' straightforward route into one country, but such an approach needs to be globally agreed and accepted to facilitate trade between all countries which can, and does take a considerable amount of time. A body of standards is under development to support commerce within the regenerative medicine fields. Terminology guides have been published by ASTM International [50] and BSI [51] which are designed to ensure consistent use of terms within the standards and the community at large. BSI have also published a guide to commercialising regenerative medicine products which is regularly updated to reflect current guidelines [52].

Another factor that needs to be considered in the commercialisation of a successful lab-based product is how to scale-up production whilst maintaining the consistency of the material. This process is usually achieved in steps via the establishment of a pilot plant, following a well-established Good Manufacturing Practice (GMP) route. Quality control

protocols are required to ensure product consistency from batch-to-batch and within a batch of material. Products derived from natural sources need to be checked for the presence of adventitious agents such as endotoxins and pathogens. Guidance on the test requirements can be found on the websites of the regulatory bodies, e.g. the FDA and the European Medicines Agency. Other, perhaps less obvious issues that need to be addressed include selection of appropriate packaging which may require hermetic sealing, UV barriers, oxygen resistant films to maximise the shelf-life of the material, identification of optimal storage conditions and an awareness of what happens should these not be available. Shipping conditions may also be relevant i.e. the packaging may need to be designed such that the product is prevented from freezing or overheating depending on the mode of transport, country and season, perhaps with the inclusion of recording equipment or an appropriate tell-tale sensor. It is also important to ensure that the product can be used under a broad range of conditions to account for variations in parameters such as local temperature and pH as well as potential processing by a range of users, some of whom may be less diligent in complying with the supplied protocol.

METROLOGY NEEDS FOR PRODUCT TRANSLATION

As new hydrogel products approach market readiness it is timely that work is carried out to develop protocols for assessment of product safety and effectiveness, guidelines on test methods for this evaluation and techniques for validation of manufacturing processes. Moreover, there is a need to develop the metrological framework under which emerging hydrogel products will be assessed. Here care needs to be taken in the preparation of protocols particularly as hydrogels are intrinsically difficult to work with due to their high water content, structural inhomogeneities, mechanical weakness and susceptibility to dehydration. Moreover, selection of candidate test methods should involve an assessment of what impact operator variance, sample preparation and the length scale samples are observed at will have on the quality of the resultant data. This section will identify key attributes linked to hydrogel safety and effectiveness and describe protocols for their assessment.

Attributes Affecting Safety & Effectiveness

Table 1 outlines the key attributes which affect hydrogel safety and effectiveness. Sterility, toxicity and the immune response relate to hydrogel safety. In the current context, sterility refers to the absence of viable microbial agents and can be achieved either by producing the hydrogel under sterile conditions or sterilising the product post manufacture through methods such as heat treatment or exposure to ionising radiation. Care should be taken to ensure that exposing hydrogels or their polymeric precursors to ionising irradiation does not alter the polymer chains in any significant way [53]. Irradiation can cause scission of the chains which obviously reduces the molecular weight which may have an impact on degradation rates and/or mechanical integrity. Ionising radiation can also initiate chemical cross-linking of polymer chains. Exposure to ethylene oxide is not a preferred method for sterilising hydrogels because of the problem of ensuring that the carcinogenic residuals are

removed. The term ‘toxicity’ covers cytotoxicity, genotoxicity, carcinogenicity and reproductive toxicity and is fundamental to hydrogel biocompatibility [54-58]. The immune response of a hydrogel must also be assessed to assure safety. Entities that may elicit an immune response include microbially produced endotoxins, degradation products and leachables as well as unreacted monomers [58]. Attributes that are linked to both safety and effectiveness include bioactive agent release, mechanical performance and osmotic stability. The release profile of bioactive agents needs to be tightly controlled and robust as an overdose or insufficient delivery is obviously undesirable and potentially unsafe. The mechanical properties of hydrogels should ideally be compatible with those of the surrounding tissues to avoid localised irritation or mechanical damage and may lead to failure of the product if it is load bearing. The osmotic susceptibility of the hydrogel should also be assessed under the same environmental conditions as those encountered in vivo to avoid any unforeseen changes in performance due to movement of solvent either into or out of the gel. Generally speaking cell motility, its setting time and degradation rate are all primarily linked to the effectiveness of hydrogel products. Care should be taken in selecting appropriate test methods to ensure reproducible and reliable measurements are made. The next section will detail possible protocols for assessment of safety and effectiveness.

Table 1. The relationship between different attributes of a hydrogel and safety/effectiveness issues

ATTRIBUTE	SAFETY	EFFECTIVENESS
Sterility	✓	
Toxicity	✓	
Immune Response	✓	
Bioactive Agent Release	✓	✓
Mechanical Properties	✓	✓
Osmotic Stability	✓	✓
Cell Motility		✓
Setting Time		✓
Degradation		✓

Protocols to Assess Safety and Effectiveness

A discussion of protocols for implementation of test methods suitable for determining key hydrogel attributes linked to safety and effectiveness is given below.

Sterility

Sterility can be assessed by determining the population of viable microorganisms present following incubation of a hydrogel or of microorganisms removed from a hydrogel in an initially sterile growth medium that is known to support microbial growth. In order to characterise the number of viable microorganisms techniques outlined in standard guidelines for sterility assessment of medical devices could be adapted which involve characterisation of the microorganism population based on the use of staining techniques, cell morphology,

colony morphology, assessment of the biochemical composition of the media or gene sequencing (ISO 11737 [59]).). The type of body contact (e.g. blood, skin) the hydrogel will have will need to be determined in order to identify the most suitable test for sterility (ISO 10993-1 [60]). The sterility of hydrogels can to some extent be managed through careful consideration of the main sources of contamination and may be overcome through the application of good manufacturing practice and with special attention to handling and the local environment.

Toxicity

Although specific test methods exist for determining the cytotoxicity, genotoxicity, carcinogenicity and reproductive toxicity of substances they generally involve exposure of a tissue model to the product and assessment of any adverse biological responses. Guidelines listed in ISO 10993-5 provide advice on methods for assessment of cytotoxicity for medical devices which includes determining changes in cell morphology and the use of cytochemical stains. [55]. Genotoxicity is primarily assessed by looking for chromosomal aberrations, advice on specific test methods for medical devices can be found in ISO 10993-3 [56]. Protocols designed to assess the carcinogenic response of materials need to take into consideration the effects of lifetime exposure of the model to the hydrogel, thus, both gross and microscopic assessments of tissue are required. If reproductive toxicity is considered to be a potential adverse effect, particularly in the case of resorbable hydrogels that contain leachables, then testing should be carried out to assess the incidence of in utero death, growth retardation and structural malformations (OECD 421 [61]).

Immune response

The potential for an adverse response of the immune system can be evaluated by considering the nature of the body contact (e.g. blood, skin [60]) with the hydrogel and the duration of this contact. Adverse immune responses can occur at the site of use or at some distant site especially in the case of resorbable hydrogels, those that contain leachables or unreacted monomer and those that produce degradation products [54, 57, 62, 63]. Assessment of the immune response typically involves the use of animal models, which if successful are followed by clinical trials. In both instances clinical observations and pathology (e.g. haematology, biochemistry, histopathology, urine analysis) are used to obtain information on the immune response and statistical analysis is performed on resulting data to evaluate its significance. Specific guidance on test methods, animal welfare and protocols to consider the effects of repeated exposure for medical devices that could be adapted for use with hydrogels and can be found in the ISO 10993 series [54-57, 60, 62-64].

Bioactive agent release

There is a wide range of bioactive agents that can be incorporated in hydrogels. The agents can range from synthetic drugs to proteins. As a result, there is also a diverse range of molecular weights, molecule shapes and sizes as well as flexibilities. Therefore, it is particularly important when characterising the ability of a hydrogel to support the release of bioactive agents that a suitably wide length scale of investigation is used. In this case, measurement protocols should recommend studies to be carried out in conditions that mimic the in vivo environment. In practice, hydrogels could be placed in a solution that matches the

physiological conditions of the site of use and the biochemical composition of the solution analysed over time. For online monitoring optical spectroscopy [33, 65] could be used, however, biochemical assays of aliquots of the solution could also be performed off line.

Mechanical properties

One relatively simple approach to investigation of hydrogel mechanical properties is penetration testing. Penetration tests involve measurement of the force required to move a probe a defined distance into a sample constrained in an open-ended container. For some hydrogels these tests can be carried out using commercially available texture analysers which are widely used in the food industry [66]. It is noted, however, that texture analysers may not be able to resolve small probe forces in the case of very weak hydrogels. Storage and loss modulus as well as creep or strain relaxation data can be obtained from rheological measurements of hydrogel samples sandwiched between a cone and plate or parallel plate geometry [67]. There are practical difficulties in making such measurements that include slippage of the plates, flow of material, and dehydration. Most of these issues can be overcome with careful experimental design i.e. the use of roughened plates, although there is some discussion as to what impact such an approach has on the measured values. Acoustic methods have been used to determine mechanical properties if [68-70] the hydrogels are too weak for conventional rheological or mechanical testing. Tensile or compression testing can be used to investigate more robust hydrogels under either static (e.g. creep recovery testing) or dynamic conditions as well as either in constrained or unconstrained geometries. Care must be taken, however, in mounting of samples. In the case of tensile testing, clamping of the sample can be difficult (e.g. it may be necessary to use pressure clamps). It should also be recognised that these materials are highly viscoelastic and therefore the complex modulus will have an innate rate dependence which can be a severe complicating factor when comparing different techniques e.g. acoustic with creep measurements.

Osmotic stability

Lack of in vivo osmotic stability can be assessed by looking at changes in the dimensions and/or wet weight of a sample immersed in a comparable in vitro environment over time [71]. Tracking changes in hydrogel shape can be done by using video microscopy, ultrasonic techniques and potentially atomic force microscopy, although there may be problems with locating the surface of the gel. Experimentally such measurements can be challenging due to lack of contrast, however in most cases such limitations can be overcome by using, e.g., dyes or reflective powders. Measuring the change in conductivity of hydrogels immersed in fluids has also been used to track osmotically induced changes in the fluid content of the matrix [72].

Cell motility

Cell motility in a hydrogel can be assessed by using a modified Boyden cell [73] where the hydrogel is placed between the two chambers; one chamber contains a chemical agent which will promote cell migration and the other contains cells. The number of cells that pass through the hydrogel over time can be determined indirectly by measuring the concentration of cells in each chamber. Alternatively, measurement of cell occupancy and distribution within a hydrogel matrix can be followed using imaging techniques such as confocal

microscopy, fluorescence microscopy and magnetic resonance imaging. For offline image analysis the hydrogel can be fixed and microtomed for histological examination.

Setting time

Setting time can be investigated using simple tests such as the tube inversion test in which a test tube is inverted periodically and the setting time determined to be the time at which inversion of the tube does not result in movement of the hydrogel [74]. Such a method, however, is not suitable for systems that set rapidly. For most hydrogels optical methods can be used to determine setting time as both optical turbidity and light scattering increases significantly at the gel point [75-77]. Alternatively the setting time can be ascertained from changes in mechanical properties, such as the storage and loss modulus [67]. These parameters can be determined from rheological measurements using, e.g., a cone and plate rheometer or from acoustic methods [68-70]. A complementary approach to these methods is to track the frequency dependent complex permittivity and conductivity using dielectric spectroscopy [78, 79].

Degradation

Degradation of hydrogels can be tracked by monitoring changes in the molecular weight of the matrix, the progressive increase in the concentration of degradation products or mass loss of the dried matrix. All three approaches are complementary. Changes in mechanical integrity can also be used to monitor bulk degradation but these can be difficult to do if the gel becomes too weak to handle. On a smaller scale changes in the chemical structure of the hydrogel can be probed using infra-red spectroscopy [33, 65], Raman spectroscopy [40, 80] and Nuclear Magnetic Resonance spectroscopy [80, 81]. Thus, in selection of a suitable test method to assess hydrogel degradation it is important to determine the required length-scale of investigation.

The above section has identified candidate test methods for assessment of hydrogel safety and effectiveness. In addition to protocols specific to each test method, consideration of the geometry of the sample, the volume of the fluid it is contained in and whether test are carried out under static or dynamic conditions also need to be made as these can strongly affect the experimental findings. Some consideration should also be given to the buffering capability of the medium in which the gel samples are housed, particularly if the material degrades through hydrolysis, and the presence of microenvironments within the matrix itself. Further, improvements to current measurement techniques could also be implemented to enhance the robustness of measurements, e.g. to variability in sample loading in rheology, and in order to reduce the number of methodologies required to fully characterise the hydrogel e.g. by extending the length scale of investigation of measurement techniques.

CONCLUSION

New developments in polymer synthesis are enabling a wide range of responsive hydrogels to be designed and manufactured with almost tailored geometrical features. These materials have excellent potential in the fields of regenerative medicine and advanced drug

delivery. A key challenge faced by researchers and companies active in these fields is characterisation of hydrogels. This involves consideration of:

- Which attributes or properties need to be determined?
- What are the recommended techniques for determining these attributes?
- What are the criteria that need to be considered in choosing test methods?
- How should the data be analysed and reported?

This information is required, not only to optimise the design of the materials used to produce novel hydrogels but also to ensure that any scale-up operation is viable and to generate quality control metrics. There is also a need for these materials to comply with regulatory demands which are still under development. Development of these regulations will benefit from the formulation of internationally agreed standards providing guidance on hydrogel characterisation. Test methods and experimental protocols outlined in this chapter have highlighted important issues that need to be considered in the establishment of such international standards that are required to be addressed in order to maximise the value of characterisation data. Overall, it is concluded that improved clarity in hydrogel metrology will expedite the translation of emerging hydrogel therapies.

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Chapter 9

METAL ION RELEASE FROM THE BASE Co•Cr•Mo, Ni•Cr, AND NOBLE Au•Pt DENTAL ALLOY INTO THE BUFFERED SOLUTIONS OF DIFFERENT COMPOSITION AND PH VALUE

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ABSTRACT

Objectives: The prevailing biological conditions in the human oral cavity induce the release of trace element (TE) metal cations from the dental casting alloys. We studied the release of metal ions (Al, Ag, Au, Ca, Cd, Co, Cr, Cu, Mg, Mo, Ni, Pd, Pt, Ti, and Zn) from three most common dental casting alloys, two base and one noble: Co•Cr•Mo, Ni•Cr, and Au•Pt alloy into three buffered biological solutions simulating the composition and pH of respective saliva, acidity, and dental plaque.

Methods: The commercially available Co•Cr•Mo, Ni•Cr and Au•Pt alloy specimens were soaked in pH 6.0 phosphate buffer (*Saliva*), 3.5 pH phosphate buffer (*Acid*) and pH 3.5 mixture of lactic, formic and acetic acid (dentobacterial *Plaque*), and incubated at 37 °C for 1, 2, 3, 4, 5, 6, 7, 14, 21, and 30 days. Six samples (n = 6) of every solution were prepared for every time period. Inductively coupled plasma atomic emission spectroscopy was used for analysis of the released elements.

Results: The concentration of the trace elements (TE) released from soaked dental metal alloys in various solutions of different pH are expressed in $\mu\text{g}\cdot\text{L}^{-1}$ as Mean values (SD). Average detectable amounts of TE from the Co•Cr•Mo dental alloy were released into: *Plaque* Co 502 (412), *Acid* Cr 589 (84), *Plaque* Fe 180 (43), *Acid* Ni 80 (96), and *Acid* Zn 87 (56). The manufacturers did not indicate the presence of Fe, Ni, and Zn. Average detectable amounts of TE from the Ni•Cr dental alloy were released into: *Acid*

Co 347 (394), *Saliva* Cr 397 (491), *Saliva* Fe 248 (257), *Saliva* Ni 542 (669), and *Plaque* Zn 96 (51). The manufacturers did not indicate the presence of Co and Zn. Average detectable amounts of TE from the Au•Pt dental alloy were released into: *Acid* Cr 895 (14), *Plaque* Cu 113 (79), *Plaque* Zn 207.4 (25.3), and *Acid* Fe 150 (43). The manufacturers did not indicate the presence of Cr and Fe. ANOVA revealed the significant effect of alloy composition, the respective buffer solution (*Saliva*, *Acid*, *Plaque*), time interval (*1 to 30 days*), and interaction on ion release ($P < 0.001$ for every compared parameter).

Conclusion: There was a great within the buffer solution variability in metal ion release from the same dental alloy indicating their inhomogeneity. The base metal alloys virtually released more ions than the Au•Pt alloy, however their effective surface area was larger than that of noble alloy. The ion release from an alloy doesn't necessarily correlate with the abundance of the element in the alloy. Rather, there is a selective dissolution so that the elements that are present in alloys only in traces can be released from them in larger amounts. The concentration of the major essential TE of Cu, Fe and Zn released from base dental alloys was below the Recommended Dietary Allowances (RDA's), whereas the leaching concentrations of the allergogenic Co, Cr, and Ni were substantial. Considerable amounts of Ni was released from Ni•Co dental alloy, whereas undeclared Co from the Ni•Cr alloy and undeclared Ni from the Co•Cr•Mo alloy may have both local and systemic, especially allergenic, adverse effects. The undeclared chromium from noble Au•Pt dental alloy appears to be responsible for the contact allergy thus far attributed to the gold.

Key words: Co•Cr•Mo dental alloy, Ni•Cr dental alloy, Au•Pt dental alloy, metal ion release, buffered solutions, saliva, plaque, acid.

1. INTRODUCTION

Any material is subjected to some degree of corrosion depending upon the environment. Similarly, all dental materials that permanently stay in the oral cavity, like alloys, ceramics, polymers and cements, are subject to the biocorrosion and would release their composite minerals, metallic, and/or organic compounds, that may induce a wide spectrum of adverse biological reactions (1-6).

Dental casting alloys are widely used for applications that are placed in the oral cavity bringing them in contact with oral tissues and oral fluids for many years. However, dental casting alloys have physical, chemical, and biologic properties that exceed other classes of materials for a long-term use in oral cavity. The selection of the appropriate dental casting alloy is essential for the long-term success of dental prostheses, and with the development of many new alloys the selection process has become increasingly complex. In the early years of a dental casting alloy applications most dental alloy compositions were based on gold, which is known to be especially resistant to corrosion (7-9). Advances in biomaterials research over the past 30-50 years, and the increase in the price of gold in the 1970s, early 1980s, and today led to diversification of the metals used in dental alloys, so that now a day typical alloys are based on gold, palladium, silver, nickel, cobalt and/or titanium (7-9). Thus, practitioners can choose among numerous alloys of different compositions, often without regard to their biological properties. A number of properties including yield strength, hardness, elastic

modulus, microstructural phases, grain size, corrosion performance, coefficient of thermal expansion, oxide color, and melting range are relevant to the proper selection of an alloy for a given clinical problem (7-11). However, the single most relevant property of a casting alloy relative to its biologic safety is the corrosion, since systemic and local toxicity, allergy, and carcinogenicity all result from elements in the alloy being released into the mouth during the process of corrosion (7-11).

Today, dental alloys must demonstrate biocompatibility prior to their human use by conducting proper testing. These testing involves the following major steps proposed by the ANSI/ADA Document No. 41: (a) Citotoxicity testing that valuates cell death in L929 or HeLa cell cultures exposed to the alloy, hemolysis testing in rabbit blood and mutagenicity testing conducted according to the Ames test, (b) Corrosion testing after static immersion test that is performed by immersing samples in an acidic solution at 37⁰ C for seven days and the residual solution is analyzed for metal ion release, and (c) Tarnish testing of cyclic immersion where the samples are dipped into solution of sodium sulfide hydrate for 10 to 15 seconds every minute and then visually analyzing with regard to color, reflectivity and roughness (12). Metal testing should also be included in that battery since many adverse reactions to released metal ions have been reported in the recent literature (13-22).

When dental alloy is inserted into the oral cavity, it is in contact with saliva. Indeed, the electrochemical conditions created in oral cavity promote the release of dental alloy's metal ions into saliva (1-11). Equally, organic and/or inorganic acids from food decrease the local pH and promote metal ion release, as do the low pH values produced by dental plaque (4, 23-27). Furthermore, microparticles are abraded from metallic restorations due to wear (4, 28-30). Some solutions like oral disinfectants, tooth bleaching agents, fluoride agents, hydrogen peroxide, different tooth-pastes, tooth brushing, alcohol, acid beverages, etc. promote ion release (1-11, 31-34). The released ions are locally and systemically distributed and may play a role in the etiology of oral and systemic pathological conditions (10). Allergic reactions due to metallic dental restorations have been documented. Nickel has especially been identified as being highly allergenic (4,10, 34-36). Moreover, the literature revealed that metal cations not being declared by the manufacturer have also been released from dental alloys (23, 25-27).

Contradictory data have been reported regarding the local and systemic health effects of dental casting alloys and their released metal ions. Therefore, it is important to elucidate the release of metals from dental alloy restorations in the oral cavity environment.

2. OBJECTIVES

To study the release of metal ions (Al, Ag, Au, Ca, Cd, Co, Cr, Cu, Mg, Mo, Ni, Pd, Pt, Ti, and Zn) from the two base alloys, i.e., Co•Cr•Mo, and Ni•Cr, and the high noble gold•platinum (Au•Pt) dental alloy under the different conditions of acidity that may be found in the oral cavity.

3. MATERIALS AND METHODS

3. 1. Clean Laboratory

All the samples were prepared and analyzed in the clean laboratory with an air work bench at the Institute for Geology, University of Zagreb, Croatia.

3. 2. Chemicals

All the chemicals used for the analytical work with inductively coupled plasma atomic emission spectroscopy (ICP-AES) were of the highest analytical quality (Suprapur, Merck, Darmstadt, Germany).

3. 3. Glassware Washing

All the glassware was soaked in 10% (v/v) nitric acid (Suprapur, Merck, Darmstadt, Germany) for 24 hours and thereafter rinsed five times in the re-distilled deionised water (DDW).

3. 4. Dental Alloy

Commercially available gold•platinum dental alloy (18+8, Noble Metal Refinery, Zagreb, Croatia) came as a standard 8.0 x 6.5 x 1.0 mm leaf with a 133 mm² effective exposure surface. Commercially available samples of Co•Cr•Mo alloy (WIRONIT^R, BEGO, Germany) and Ni•Cr alloy (WIRON 99®, BEGO, Germany) came as standard rollers (8.0 mm in diameter and 15.8 mm in height) with a 498 mm² effective exposure surface. The declared metal composition of the alloys is shown in Table 1. To preclude the bacterial growth contamination, the alloy samples were washed with Alconox soap (Alconox Inc, Alconox, New York, NY, USA), rinsed with the Deideonised and Demineralised Water, further soaked for 20 min in alcohol, again rinsed twice with DDW in a laminar flow hood, and dried at 60⁰C for 24 h.

3. 5. Immersion (Extraction) Solutions

Three different buffered immersion solutions were prepared for metal extraction: (1) pH 6.0 phosphate buffer, to resemble pH of saliva (*Saliva*) (37); (2) pH 3.5 phosphate buffer to resemble extreme conditions (*Acid*), and (3) pH 3.5 mixture of 0.1 M lactic acid, 0.1 M sodium chloride, 0.1% acetic acid, and 1.0% formic acid; (*pro analysis*, Kemika, Zagreb, Croatia) to resemble conditions under dentobacterial plaque (*Plaque*). Natural fresh saliva has a pH 5.7-7.0. Bacteria in the active dental plaque generate a powerful pH 4.0, or even lower, tooth mineral destroying mixture of lactic, formic, acetic and other metabolic acids (38).

Table 1. Declared and detected trace elements (Au/Pt, Co/Cr/Mo and Ni/Cr alloys)

Alloy	Metal	Declared (%)	Detected
Au•Pt alloy	Gold (Au)	75.0	Not detected (ND)
	Platinum (Pt)	8.0	ND
	Silver(Ag)	9.5	ND
	Copper (Cu)	5.1	Detected
	Brass (Cu+Zn)	1.5	Detected
	Zinc (Zn)	Declared in brass	Detected
	Other elements		Detected
	Iron (Fe)	Undeclared	Detected
	Chromium (Cr)	Undeclared	Detected
Co•Cr•Mo alloy	Cobalt (Co)	64.0	Detected
	Chromium (Cr)	28.65	Detected
	Molybdenum (Mo)	5.0	Detected
	Silicium (Si)	1.0	ND
	Manganese (Mn)	1.0	ND
	Carbon(C)	0.35	ND
	Other elements		Detected
	Zinc (Zn)	Undeclared	Detected
	Iron (Fe)	Undeclared	Detected
	Nickel(Ni)	Undeclared	Detected
Ni•Cr alloy	Nickel(Ni)	65.0	Detected
	Chromium (Cr)	22.5	Detected
	Molybdenum (Mo)	9.5	Detected
	Silicium (Si)	1.0	ND
	Niobium (Nb)	1.0	ND
	Cesium (Ce)	0.5	ND
	Iron (Fe)	0.5	Detected
	Carbon(C)	<0.02	ND
	Other elements	Detected	Detected
	Cobalt (Co)	Undeclared	Detected
	Zinc (Zn)	Undeclared	Detected

3. 6. Metal Soaking

Individual 15 ml glass tubes with plastic stopper (culture tubes with GL thread, AR-Glass®, Brand Gmbh, Wertheim, Germany) were used. Six replicates of each alloy were immersed in 7 ml of *Saliva*, *Acid*, and *Plaque* extraction solution, respectively, and incubated at 37 °C for 1, 2, 3, 4, 5, 6, 7, 14, 21, or 30 days.

3. 7. Metal Analysis

The amount of metal released into the immersion solutions was assessed with the inductively coupled plasma atomic emission spectrometer (ICP-AES, Jobin Yvon 50 P,

Horiba group, Longjumeau, France). We used certified multielement stock standards for the quality control and to avoid matrix induced interference (Spex, Metashem, NY, USA). The multielement calibration standards of 0, 1, 5, 10, 25, 50, 500, 1000 and 5000 $\mu\text{g}\cdot\text{L}^{-1}$ in every immersion (extraction) solution were prepared from the above stock standard (100 $\text{mg}\cdot\text{L}^{-1}$).

3. 8. Reagent Blanks

The blank immersion solutions of *Saliva*, *Acid*, and *Plaque* for all the analyzed elements were prepared in the same glassware, kept at the same temperature for the same period of time as were the immersion solutions in which the Au•Pt, Ni•Cr, and Co•Cr•Mo dental alloys were soaked. No metal was detected in the reagent blanks.

3. 9. Detection Limits

The detection limits were determined from the matrix-matched multielement calibration standards prepared from the certified stock standard (see above). The accurate detection limits for Al, Ca, Cd, Co, Cr, Cu, Mg, Ni, V, Ti, and Zn were 10 $\mu\text{g}\cdot\text{L}^{-1}$ and that for Ag, Au, Mo, Pd, and Pt were 100 $\mu\text{g}\cdot\text{L}^{-1}$. Some signal may be perceived even below the already stated accurate detection level (perceptible).

3.10. Statistics

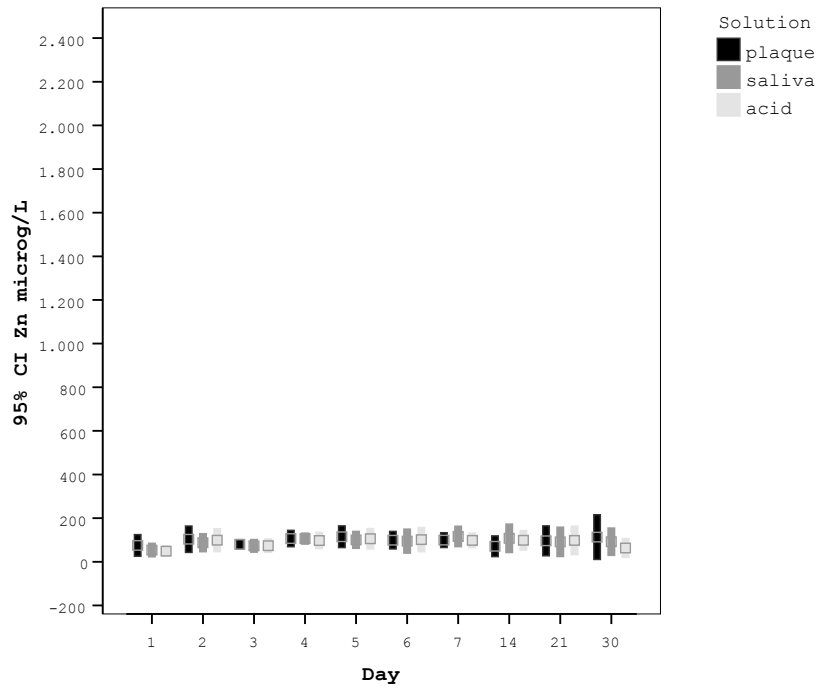
The results were expressed as a Mean with standard deviations (SD) (Table 2, Figure 1 Parts A,B and C). The overall difference between the alloys, buffers and exposure time was assessed with MANOVA, using the Scheffe post-hoc with the significance set at $p < 0.05$. All analyses were made using a statistical package SPSS 14 for Windows (Chicago, Illinois, USA).

4. RESULTS

4.1. Noble Au•Pt Alloy

Most of the principal metal constituents declared to be present in the Au•Pt dental alloy did not leach into either of the immersion solutions or leach below the detection limit of the ICP AMS. However, some of the undeclared metals did leach in the amounts well above their detection limit (Table 1). Thus, copper, zinc, iron and chromium leached from the Au•Pt alloy, whereas no traces of gold, platinum, or silver were detected, regardless of how long the alloy was immersed in either of the three extraction solutions. Some zinc might have been expected due to the declared presence of brass, an alloy of copper and zinc, but the presence of iron, and especially chromium, could not be anticipated from the declared Au•Pt alloy composition.

A/ Ni•Cr dental alloy
Zn



Fe

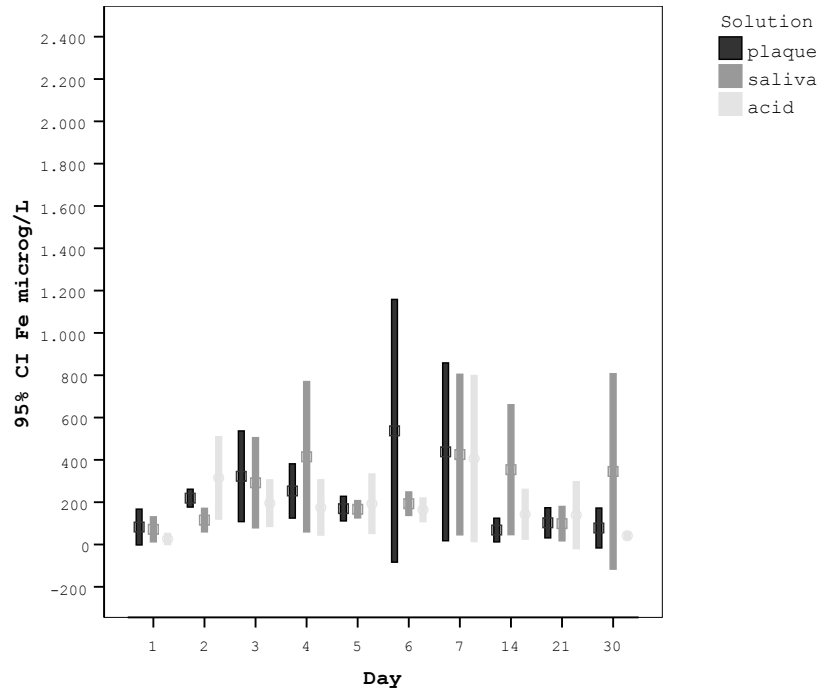
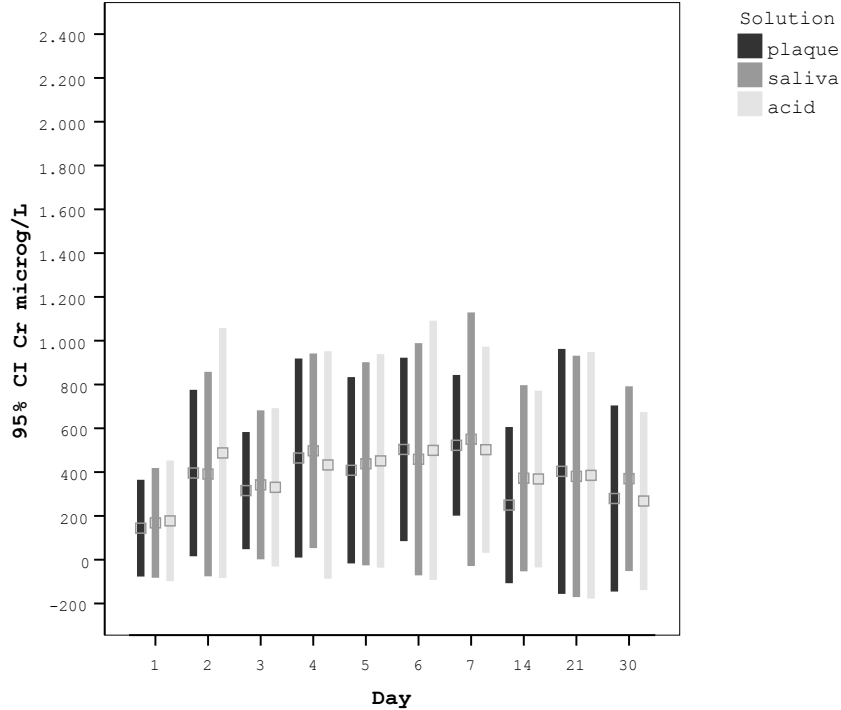


Figure 1. (Continued)

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Cr



Co

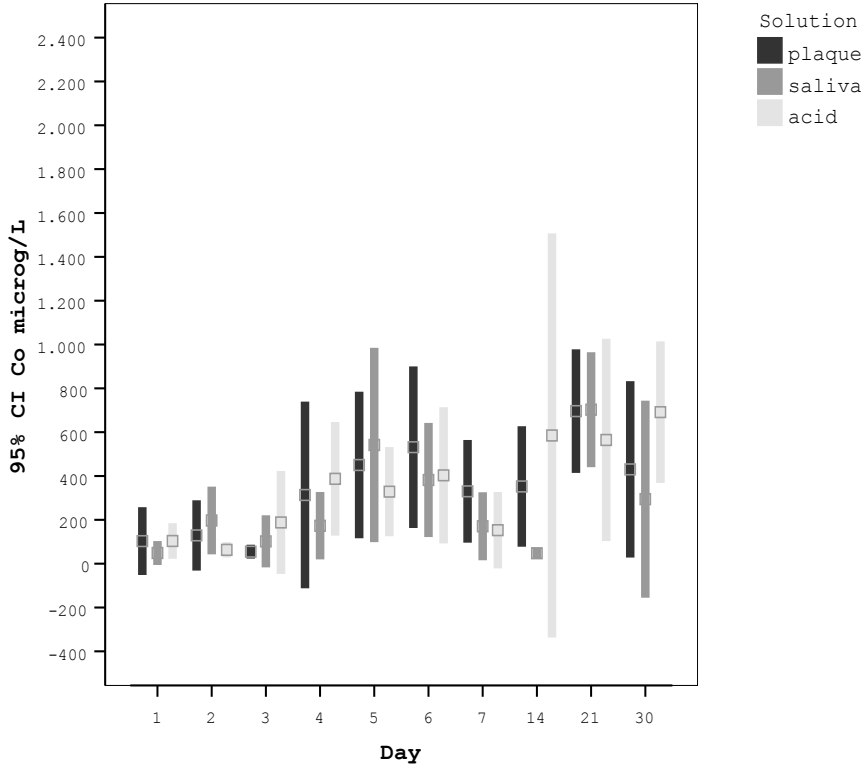
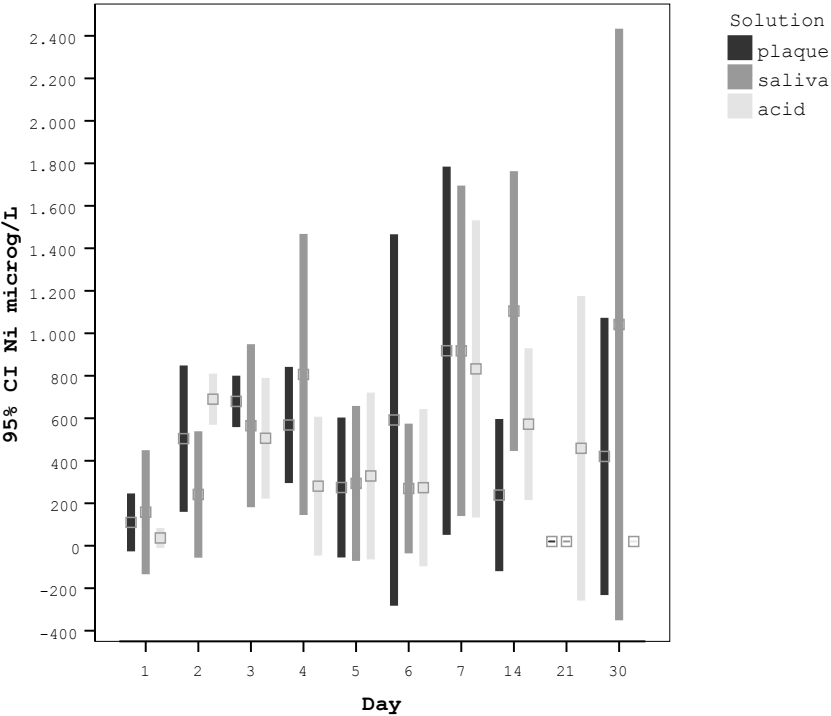


Figure 1. (Continued)

Ni



B/ Co•Cr•Mo alloy
Zn

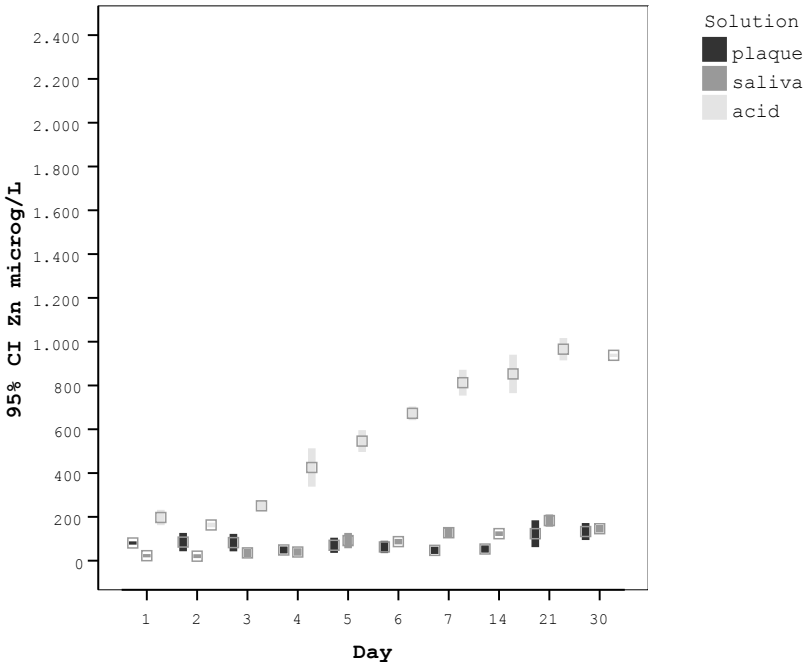
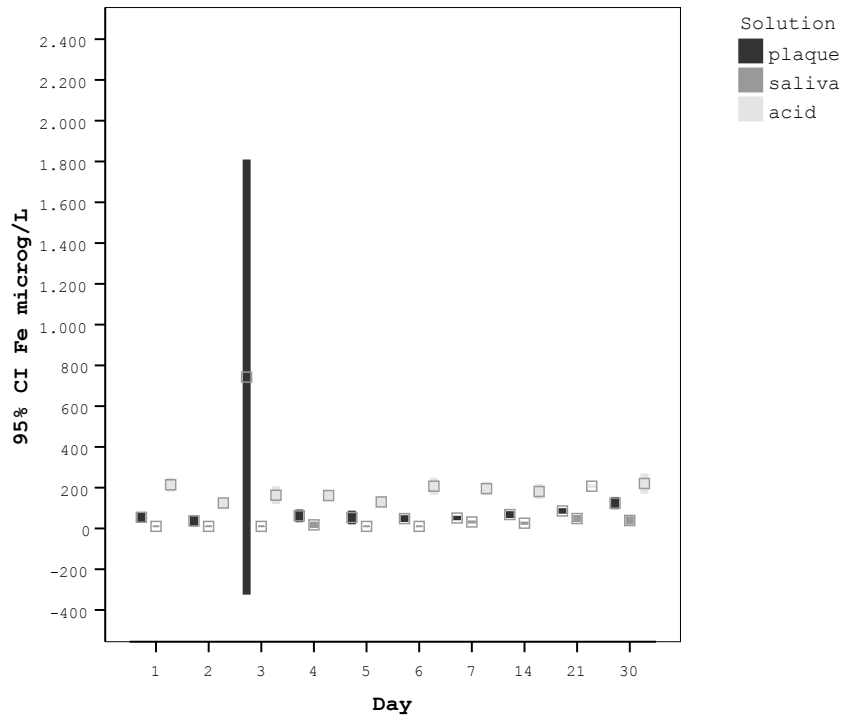


Figure 1. (Continued)

Fe



Cr

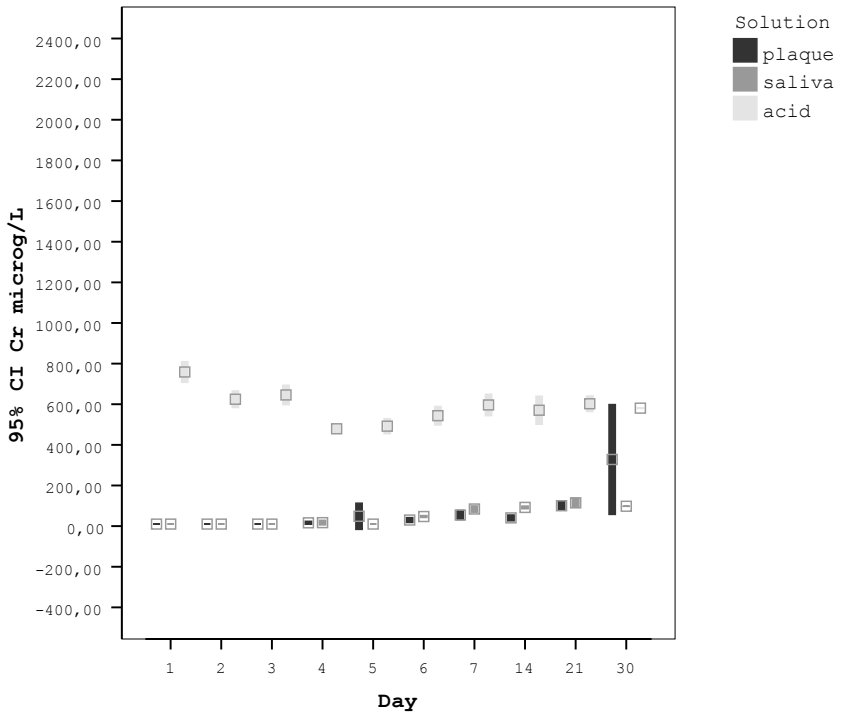
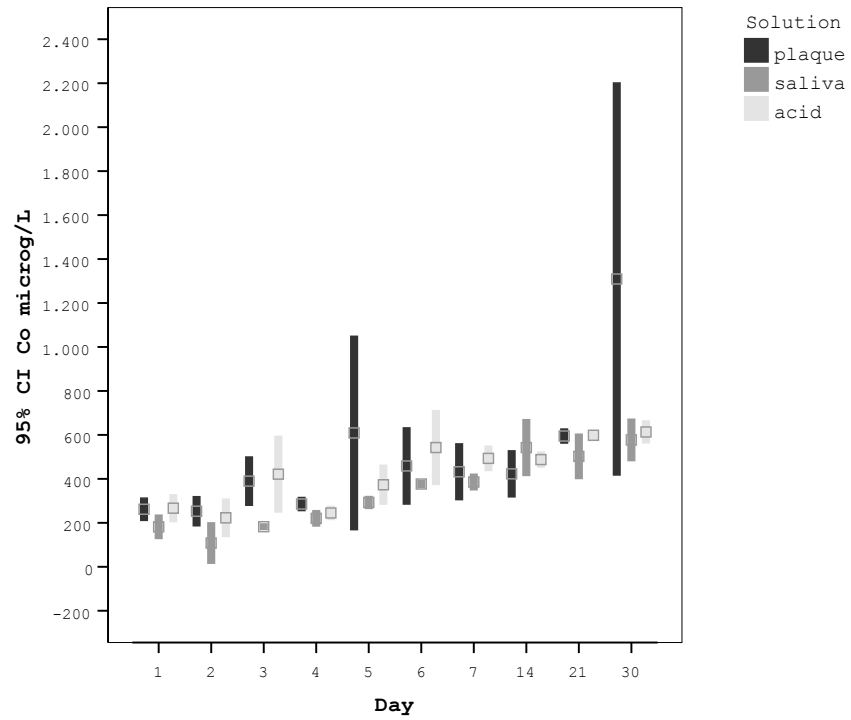


Figure 1. (Continued)

Co



Ni

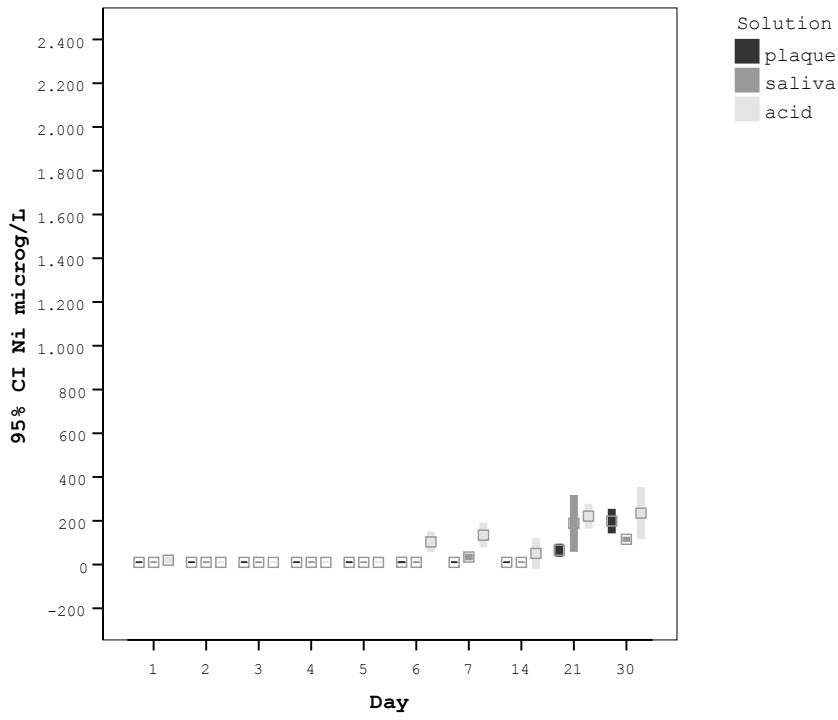
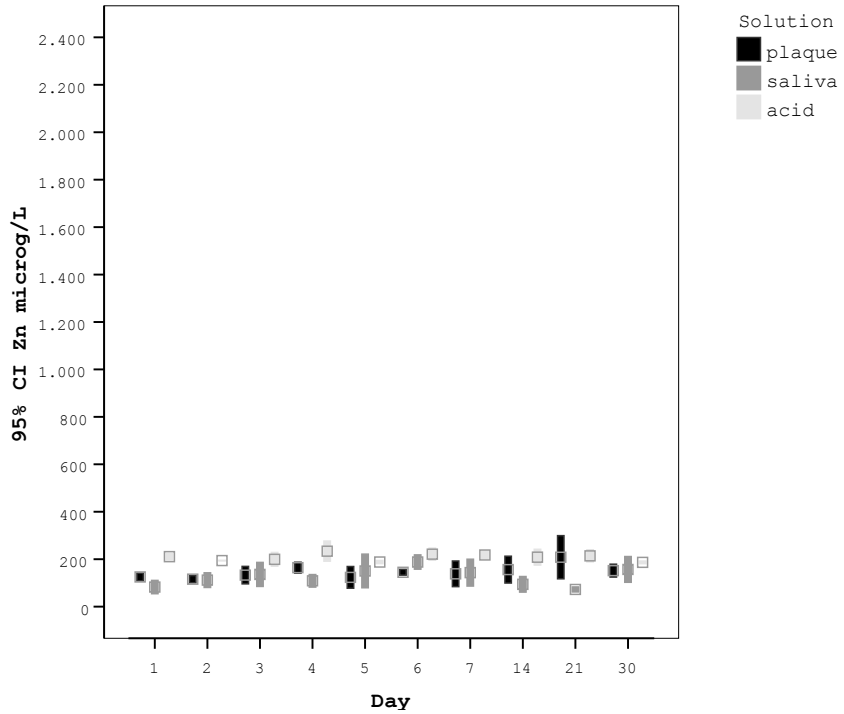


Figure 1. (Continued)

C/ Au•Pt alloy
Zn



Fe

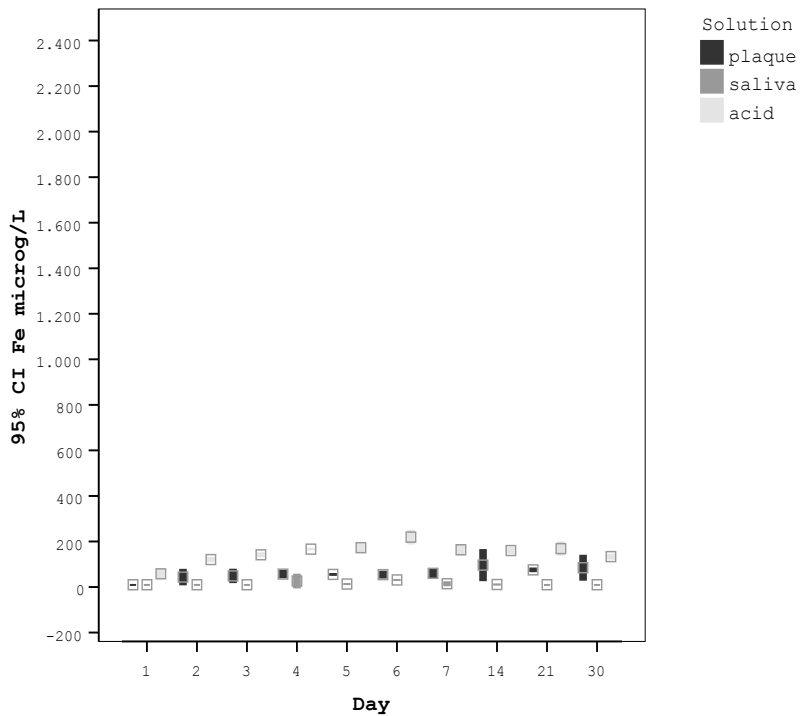
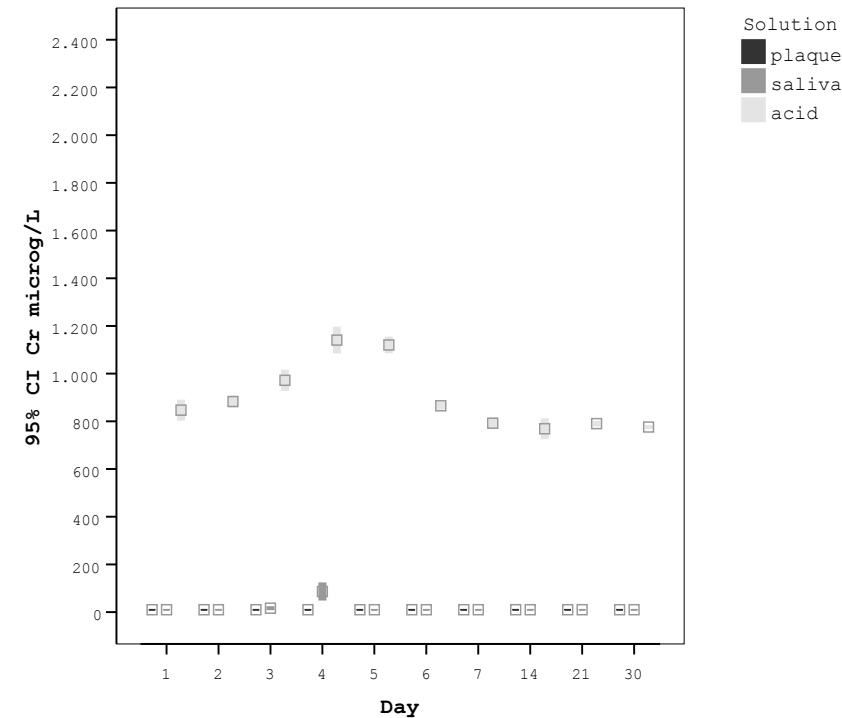


Figure 1. (Continued)

Cr



Cu

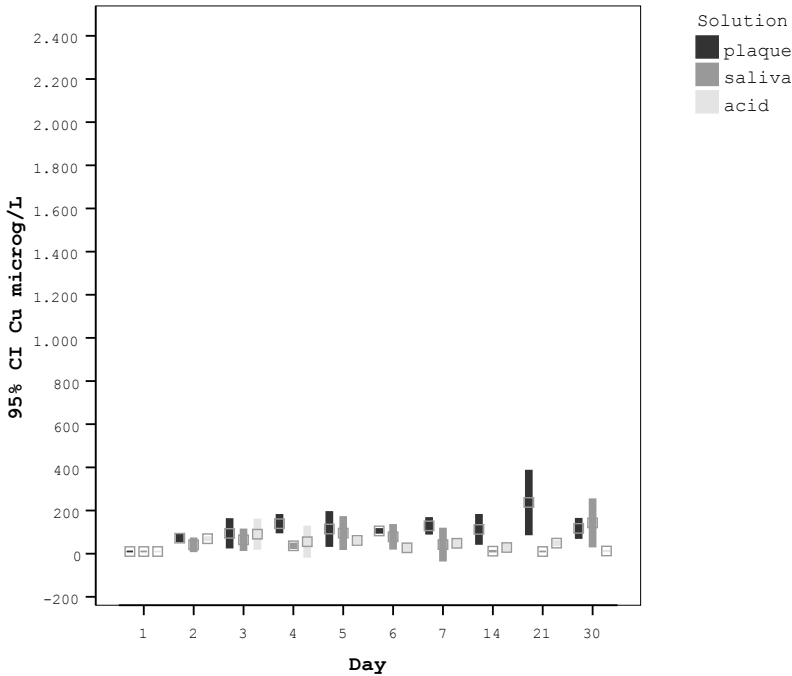


Figure 1. 95% confidence intervals of ions released from the A/ Ni•Cr, B/ Co•Cr•Mo, and C/ Au•Pt dental casting alloys immersed into the solution of *saliva*, *acid*, and *plaque* over the period of thirty days. (see Materials and methods for the details).

As evident from Table 2 and Fig. 1C, the within sample variability of results over the time line of 30 days could be quite remarkable. Zinc and copper could be accurately quantified in all the immersion solutions since Day 1 and Day 2, respectively; the copper signal during Day 1 was perceptible at the low limit of detection. Iron became perceptible in both *Acid* and *Plaque* immersion solution already on Day 1, but fully detectable (precisely quantified) on Day 2 and thereafter. However, in *Saliva* iron built up from a barely perceptible level at Day 1 to a quantifiable level by Day 4, then remained well detectable through Day 14 to fall down again to a barely perceptible level on days 21 and 30, respectively. Chromium, on the other hand, would stay at the low level of detection limit in the *Plaque* solution during the entire immersion period of 30 days. It was well detected in only a few samples on the Days 3 and 4 in *Saliva* and thereafter only perceptible for the rest of the immersion periods, but it was massive in the *Acid* ever since the Day 1. In some cases the concentration of metal in the immersion solution would slowly go up for the first few days, only to drop down almost to the day 1 level thereafter; presumably due to the surface re-adsorption of the already leached metal and/or the unhomogeneity of the analyzed alloy sample.

Table 2. Average ion release ($\mu\text{g}\cdot\text{L}^{-1}$) from the Au•Pt, Ni•Cr, and Co•Cr•Mo dental alloys (Mean with SD) during all exposure intervals. Effective surface area (ESA) in mm^2

Alloy	Element	<i>Extraction solution</i>		
Au•Pt (ESA 133)		<i>Saliva</i>	<i>Acid</i>	<i>Plaque</i>
		Mean (SD)	Mean (SD)	Mean (SD)
	Chromium	18 (25)	895 (135)	10 (0)
	Copper	53 (63)	45 (38)	113 (79)
	Iron	15 (11)	150 (43)	59 (36)
	Zinc	124 (51)	146 (47)	207 (25)
Ni•Cr (ESA 498)	Chromium	397 (410)	390 (426)	369 (361)
	Cobalt	266 (300)	347 (394)	339 (318)
	Iron	248 (257)	180 (183)	225 (273)
	Nickel	542 (669)	400 (433)	432 (502)
	Zinc	92 (46)	88 (43)	96 (51)
Co•Cr•Mo (ESA 498)	Chromium	49 (42)	589 (84)	65 (120)
	Cobalt	337 (170)	426 (160)	502 (412)
	Iron	21 (15)	180 (43)	133 (360)
	Nickel	41 (68)	80 (96)	35 (60)
	Zinc	88 (56)	582 (300)	79 (31)

Evidently, zinc, iron and chromium leached the most from Au•Pt alloy when in the *acid* (phosphate buffer pH 3.5), whereas copper leached the most when under the *Plaque* conditions (organic acid mixture, pH 3.5). In contrast, zinc and iron leached the least in the *Saliva* (phosphate buffer, pH 6.0); copper in the *Acid* (pH 3.5 phosphate buffer), and chromium leach out in the *Plaque* immersion solution. The presence of undeclared electronegative zinc, iron, and chromium in the presence of the electropositive copper would

generate a high electrical potential within the oral cavity, promote the corrosion of the Au•Pt dental alloy and increase the leach out of the metals.

4.2. Base Alloys: Co•Cr•Mo and Ni•Cr

Some of the principal metal constituents declared to be present in the base Co•Cr•Mo and Ni•Cr dental alloys (Table 1) were released into either of the immersion solutions and some were not, whereas some of the undeclared metals were also released in the amounts well above their detection limit (Ni and Zn from Co•Cr•Mo alloy, Zn and Co from Ni•Cr alloy) (Table 1 and Table 2, Figure 1A).

Thus, zinc, iron and nickel leached from the Co•Cr•Mo alloy, whereas no traces of Mo some other elements were detected regardless of how long the alloy was immersed in either of the three solutions (Tables 1 AND 2, Figure 1B). Zinc, iron and chromium leached the most when in the *Acid* at pH 3.5, whereas cobalt leached the most when in either the *Plaque* or *Acid* at pH 3.5. Amounts of released zinc ions increased gradually over the time line of 30 days in the *Acid*. Similar findings were observed for Co in all the tested solutions. At the initial period of exposure the nickel ions were released in all three solutions in very small amounts ($10 \mu\text{g}\cdot\text{L}^{-1}$). The increase of Ni ion release was observed on the sixth day in *Acid*; significant release of nickel also occurred on the 21st and 30th day in saliva and plaque.

Similarly, Ni, Cr, Fe, Co and Zn were the only detected cations released from Ni•Cr dental alloy (Table 2, Figure 1A) and no trace of Mo was detected. Chromium and zinc were not declared (Table 1). The amount of released zinc ions was lower from Ni•Cr than Co•Cr•Mo alloy. The amounts of Co ion released were similar from both alloys, while the amounts Fe, Cr and Ni ions released were higher from Ni•Cr alloy.

In summary, there were no general rule on how the particular metal alloy would respond, as their release into the solution depended both upon the nature of the solution and the exposure time. Multivariate testing revealed that the type of dental alloy, the composition and pH of extraction solutions and the duration of exposure have significant impact on the metal ion release ($p<0.001$). The combination of two, or all three factors (alloy, extraction solution, duration of exposure) significantly affected the metal ion release ($p<0.001$).

The composition and pH of solutions significantly influenced the release of Zn, Cr and Co cations ($p<0.01$), but not the release of Fe and Ni ions. Duration of exposure in tested solutions significantly influenced the release of Zn, Co, Fe and Ni ions ($p<0.05$), but not Cr ions. The type of dental alloy and its composition significantly influenced ion release ($p<0.001$). Combination of Solution*Duration of exposure significantly affected the release of Zn, Fe ($p<0.01$), and Ni ($p<0.05$), but did not affect the release of Cr and Co. Combination of Solution*Alloy significantly influenced the release of Zn, Fe and Cr ($p<0.01$), but did not affect the release of Co and Ni ions. Combination of Alloy*Duration of exposure significantly affected release of Zn, F, Co and Ni ($p<0.05$), but did not affect significantly the release of Cr. Combination of Solution*Duration of exposure*Alloy significantly influenced the leach of all ions (Zn, Fe, Co, Ni), except Cr.

There was a strong interaction between the variables of the immersion solution, soaking time, type of dental alloy tested and interaction for the every detected metal, respectively (MANOVA, $p<0.0001$).

5. DISCUSSION

Ideally, the materials employed in the mouth should not react with many alkaline and acid foods, and they should not be affected by mouth fluids. The potential for corrosion is an important part in the bio-compatibility of an alloy and, since alloys used in dentistry usually contain at least four metals and often more, makes the issue of bio-compatibility a complex one (12).

High-noble alloys (gold, platinum and palladium) are stable in their elemental form. On the other hand, most base metal alloys, such as titanium and cobalt•chrome, are stable in the oral environment as a result of passive oxide layers that cover their surface. These oxide layers act as a corrosion barrier. In a Co•Cr alloy the oxide layer is only 1-4 nm thick (39). Despite the above facts, it is well documented that metal ions have been released from various dental alloys (1-11, 24-27, 32-36). Our results also showed metal cation release from all of the three tested alloys. The principal finding of our study is the presence of undeclared metal ions in all of the three tested alloys.

Average detectable amounts of TE from the Co•Cr•Mo dental alloy were released into (Mean (SD)): *Plaque* Co 502 (412), *Acid* Cr 589 (84), *Plaque* Fe 180 (43), *Acid* Ni 80 (96), and *Acid* Zn 87 (56). The manufacturers did not indicate the presence of Fe, Ni, and Zn. Average detectable amounts of TE from the Ni•Cr dental alloy were released into: *Acid* Co 347 (394), *Saliva* Cr 397 (491), *Saliva* Fe 248 (257), *Saliva* Ni 542 (669), and *Plaque* Zn 96 (51). The manufacturers did not indicate the presence of Co and Zn. Average detectable amounts of TE from the Au•Pt dental alloy were released into: *Acid* Cr 895 (14), *Plaque* Cu 113 (79), *Plaque* Zn 207.4 (25.3), and *Acid* Fe 150 (43). The manufacturers did not indicate the presence of Cr and Fe. However, individual samples tested in this study released much higher amounts of metal ions occasionally (Fig 1).

Undeclared chromium was released even under the normal pH conditions (*Saliva*, pH 6.0) from the noble Au•Pt alloy. In the Ni•Cr and Co•Cr•Mo base alloys chromium was one of the constituents and its release was expected. However, under *Saliva* conditions more Cr was released from Ni•Cr than from Co•Cr•Mo and Au•Pt alloy. *Acid* conditions caused the release of the substantial amounts of chromium from all three tested alloys, and undeclared chromium reached almost $1400 \mu\text{g}\cdot\text{L}^{-1}$ from the Au•Pt alloy. We assume that there was a selective dissolution of chromium so that the elements that were present in alloys only in traces were released from them in considerable amounts. Based on the results of this study and being aware of the allergenic potential of Cr (40), we may assume that Cr released from dental golden alloy may be responsible for allergies so far attributed to gold (25). Indeed, researchers who claimed allergy to gold like Tvinnerein et al (41) and Moller et al (42) did not provide the analytical data on other element impurity in their gold. Moreover, Linde et al. (43) did not detect any gold to be released from the gold-containing jewellery materials. Cobalt, chromium, and nickel are potent metal allergens ($\text{Ni} > \text{Cr} > \text{Co}$) that may induce local and systemic allergic reaction (44). Contact allergy to metals is a fascinating subject since many of the trace elements, including Co, Cr, and Ni, are at the same time allergenic and essential for the human health and well being (45). The Recommended Dietary Allowances (RDA's) suggest Adequate Intake (AI), or Upper Limit (UI) of any TE in human diet (46). Our results showed that upper limit of 95% confidence interval for Cr leached from Ni•Cr alloy was almost $1200 \mu\text{g}\cdot\text{L}^{-1}$, $800 \mu\text{g}\cdot\text{L}^{-1}$ from Co•Cr•Mo alloy, and almost $1400 \mu\text{g}\cdot\text{L}^{-1}$ from

the Au•Pt alloy (Cr even not declared) (Figure 1). Adequate intake (AI) of Cr is 50-200 µg/day (47). Trivalent chromium is an essential nutrient required for sugar and fat metabolism (47) and the majority of people eating typical Western diets consume less than the upper limit of the estimated safe and adequate daily dietary intake. Insufficient chromium intake is associated with signs and symptoms similar to those seen in diabetes and cardiovascular diseases. One study showed that the use of 1000 micrograms of chromium per day (five times above the upper limit of the estimated safe and adequate daily dietary intake) was highly effective in relieving many of the symptomatic manifestations of type 2 diabetes mellitus (47). Most recent evidence supports the conclusion that there is little fear of toxic reactions from chromium consumption even in higher concentrations than the daily UL (47,48).

Iron was found in the immersion solutions of all three tested alloys. The largest amounts of Fe, reaching up over 1000 µm•L⁻¹ were found in solutions with Ni•Cr alloy. Upper limit (UL) for Fe is 45 000 µgd⁻¹ (46). Iron is fortunately the essential nutrient and is usually lacking in the diet of old people, so the released Fe from dental alloys may be regarded as a dietary supplement.

Zinc was also found in small amounts in the extraction solutions from all three alloys. Acid condition caused the release of larger amount of Zn only from Co•Cr•Mo alloy, up to almost 1000 µm•L⁻¹. However, it was far below the daily dietary needs that are over 3 mg a day for older adults (47), the segment of population that usually have removable Co•Cr•Mo cast dentures. The RDA's UL is 40 000 µg/day (40 mg/day) (46).

Copper leached only from the Au•Pt dental alloy. Our results showed that the upper limit of 95% confidence interval for Cu release was less than 500 µm•L⁻¹ (Figure 1C), much lower than the RDA's UL for Cu, which is 10 000 µm/day (46).

Cobalt and nickel leached only from the two base alloys. Co was not declared in the Ni•Cr alloy, but leached as much as it did from the Co•Cr•Mo alloy. Acid buffers (*Acid* and *Plaque*, pH 3.5) caused greater release of both, cobalt and nickel.

As a component of Vitamin B₁₂, cobalt is an essential metal. Cobalt in Vitamin B₁₂ is unique by having a carbon-cobalt bond to a methyl group, (-CH₃) in its active coenzyme form (49, 50). Like nickel, cobalt hypersensitivity is expressed as contact dermatitis and asthma. Cobalt-induced dermatitis in general population is rare and its prevalence likely does not exceed 1%. However, it is often found in association with nickel sensitivity in females and chromium sensitivity in males and a prevalence of 7% of positive reactions to cobalt has been reported in eczema patients (49). Cobalt daily AI is 24 micrograms a day, and UL is 1000 micrograms/day (46), but adverse effects to Co have been reported only when consumed over 10 mg of cobalt per day as cobaltous chloride (50), which is more than 250 times higher than the current ULs.

Nickel was not declared in the Co•Cr•Mo alloy and leached in small amounts from that alloy. However, the amounts of the nickel released from the Ni•Cr alloy were substantial and exceeded 2000 µg•L⁻¹ in extraction solutions of one 30-day sample. RDA's UL for Ni is 1000 micrograms/day. The results of most recent epidemiological studies indicate that cobalt, chromium and nickel are metals which most often produce allergic responses (51,52). Peltonen noted that women were ten times more sensitive to Ni compared to men (53), and Wataha related oral inflammation to the less than 4 µg•g⁻¹ of nickel present in the tissue for 1-2 mm around the implants containing nickel (54). Analysis of the multielement profile of hair, revealed that one patient who for two years suffered from boats of fatigue, vertigo, loss of

posture control, different migrating paresthesia, gastrointestinal cramps and pain associated with diarrhoe had increased nickel hair concentrations. Subsequently was shown that the patient was nickel sensitive (45). After replacing the existing dental alloy containing nickel, all the symptoms vanished, and patient's health condition was fully restored.

The leaching of metal ions from the tested alloys in different extraction solutions was dependent upon the selected alloy, the nature of the immersion solution, and the duration of the immersion. Elements making up an alloy were not released in proportion to their percent weight, moreover some of the undeclared elements were detected. Factors influencing how much of a particular element is released are pH value, composition of the extraction solution and the duration of immersion and this is consistent with the findings of other papers (3-9,25-27, 55-57). For example, it was found that nickel was released from nickel-chromium alloys to a much greater extent in an acidic environment (56,57), which is similar to our results, especially for the 3.5 pH *Plaque* solution. The noble Au•Pt alloy used in this study was very resistant to corrosion, much more than base alloys, except for the *acid* condition and Cr ions. This can be attributed not only to the Au•Pt alloy composition, but also to the the larger effective surface area of the base alloys tested.

For chemical corrosion resistance, three classes were distinguished according to the quantity of metallic ions released. It was proposed that Class III (100-1000 $\mu\text{g}/\text{cm}^2$ week) was not acceptable (2). The results of our study are similar to Lopez-Alias at al. (58), but contrary to Denizoglu et al. (59), who found that pH significantly affected total and Co ion release, but not Ni or Cr ion release. Patients with metal restorations have also significantly greater metal content of saliva than patients without metal restorations (60).

However, the real release of ions in the oral cavity may be even the more excessive than in our experiment, since dental alloys are exposed to everyday tooth-brushing that, promotes ion release by mechanical removing the oxide protective layer (3,5,6). Dental alloys are exposed to acid beverages (although the duration of such contact can be only several seconds), and to salivary proteins, that were not the constituents of the extraction solutions as used in this study. Both proteins and cells presented in saliva can modify ion release. It was found that in subjects having artificial Co•Cr•Mo alloy hips, metal ions were released by dissolucytosis - a process where macrophages induce the release of metal ions from the metallic surface (61). Bacterial adherence accompanied with a poor oral hygiene will produce *Plaque* conditions on dental restorations, and stimulate bacteria to act like macrophages. The metal ions leached locally can be accumulated systematically in distant organs, such as liver, kidney, lung nails, hair, etc (61,62). Indeed, several weeks after the insertion of a dental alloy to the rat, the content of the dental alloy metal ions in the rat organs was increased (62).

Ni•Cr alloy, due to abundant release of Ni ions registered in this and some other studies (27,50,51), and especially in patients with hypersensitivity or poor oral hygiene should be avoided. Furthermore, Ni•Cr alloy should not be exposed in the oral cavity. This alloy is usually covered with a ceramic material, but sometimes it might be used for acrylic-veneer crowns due to its low cost and what, certainly, should not be done. In such cases it is more safe to use Co•Cr•Mo alloy.

It is pertinent to note here that we demonstrated a considerable variability in metal leach and undeclared TE. The concentration of the major essential TE of Cu, Fe and Zn released from base dental alloys was below the Recommended Dietary Allowances (RDA's)(46). The leaching concentrations of the allergogenic Co, Cr, and Ni were above RDA's in some samples incubated over 7 days.

Further studies of different dental alloys in regard to their ion release, their specific biocompatibility and possible cytotoxic effects on mucosa and adjacent tissue and distant organs, and the role of acid beverages, would be desirable.

6. CONCLUSION

There was a great within the buffer solution variability in metal ion release from the same dental alloy indicating their inhomogeneity. The base metal alloys virtually released more ions than the Au•Pt alloy, however their effective surface area was larger than of the noble Au•Pt alloy. There was a selective dissolution so that the elements that were present in alloys only in traces were released from them in larger amounts. The concentration of the major essential TE of Cu, Fe and Zn released from both base and noble dental alloys was below the Recommended Dietary Allowances (RDA's), whereas the leaching concentrations of the allergogenic Co, Cr, and Ni released from base alloys were substantial. Considerable amounts of Ni was released from Ni•Co dental alloy. Undeclared Co from the Ni•Cr alloy and undeclared Ni from the Co•Cr•Mo alloy were released. Ni, Co and Cr may have both local and systemic, especially allergogenic, adverse effects. The undeclared chromium from noble Au•Pt dental alloy appears to be responsible for the contact allergy thus far attributed to the gold.

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Chapter 10

CO-CR-MO ALLOY SURFACE FEATURES AND COMPOSITION PRIOR AND AFTER MECHANICAL POLISHING AND CORROSION IN FLUIDS SIMULATING ORAL CONDITIONS

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ABSTRACT

Introduction: It has been well-documented that metal ions are released from all dental alloys, which may be of a clinical concern and may be a potential health problem. Corrosion behaviour in oral fluids is strongly influenced by microstructural characteristics and surface properties of dental alloy.

Aim of the Study: To examine the surface of CoCrMo alloy prior and after exposure into different solutions simulating oral conditions, as well as prior and after different mechanical polishing procedures.

Materials and Methods: The cast and electropolished specimens of CoCrMo alloy (EP) (Wironit®, extra hard) were examined by SEM with EDS and by AFM prior and after immersion into two solutions simulating saliva and dentobacterial plaque for 30 days at 37 °C. The effect of polishing at the surface of CoCrMo alloys has also been studied. After electropolishing procedures (EP, 6 samples) another samples were further mechanically polished using green rubber discs and afterwards a high shine polishing paste and a rotating black brush (BB, 6 samples), or using green rubber discs, a high shine polishing paste and a rotating deer leather brush (DL, 6 samples).

Results: The AFM analysis provided usefull information about the surface morphology. AFM imaging revealed that the surface of EP specimens prior the contact

with corroding solutions was characterized by clusters of nanocrystallites forming islands emerging from the smooth undulating surface. The islands' height was in the range of 90-150 nm. Besides undulating surface and islands of nanocrystallites, some high conical crystallites and small grains can also be observed. The undulating surface was twice richer with Co than Cr, while in the clusters of nanocrystallites (islands) Co was almost equal to Cr, which is different from the declared alloy's composition. However, some crystallites were dissolved after corrosion, but some new phases (dense sprinkles of very small crystallites) appeared. The respective alloy peaks in the EDS spectrum revealed that these phases were a result of a corrosion - primarily of metal dissolution and salt formation. The presence of some elements from the corroding solution was registered at the alloy surface, as well. The effect of mechanical polishing was clearly evident at CoCrMo surface. The clusters of nanocrystallites forming islands (emerging from the undulated surface) were smoothened and/or thorn away by further mechanical polishing, while the islands' perimeter was unaltered. Mechanical polishing also produced scratches in both, BB and DL samples. The scratches were observed not only at the undulating surface, but also at the smoothed islands. The Z range (distance from the highest to the lowest peak) of EP samples revealed significantly higher values than that of BB and DL ($p < 0.01$). The highest RMS and the Ra values were also recorded in the EP samples, but not significantly different ($p > 0.05$), compared to the mechanically polished BB and DL samples.

Conclusions: AFM imaging revealed at least four different phases at the surface of CoCrMo electropolished specimens. The most dominant phases were: undulating surface and clusters of nanocrystallites emerging from the undulating surface forming islands, but some high conical crystallites and some small grain-like crystallites have also been observed. Mechanical polishing of EP samples smoothed islands of nanocrystallites, but also formed scratches at the surface, which were more prominent in DL than BB samples. During the corrosion process of EP samples probably metal salts (anions from corroding solutions) were binded at the surface as a new phase. Corrosion behaviour of CoCrMo alloys is clearly affected by pH and composition of the fluids. It is obvious that some complex mechanisms involving reactions between ionic species are dominating the interfacial processes, which in turn affect the morphology and the composition of the phases formed.

Keywords: Cobalt-chromium alloy, corrosion, SEM with EDS, surface phases and composition, AFM imaging, surface topography, effect of mechanical polishing

INTRODUCTION

Dental materials have to withstand mechanical, thermal, and chemical stresses in the patient's mouth and therefore must have a sufficient biocompatibility [1]. Consequently, the surface quality a dental alloy (the surface roughness and the surface morphology) is of utmost importance, as this determines the area of the contact surface and thus influences the corrosion behaviour and the biocompatibility, as well as microorganism and/or human cells interactions with the alloy [2]. Therefore, a complete study on surface topographical features in a broad range of scales is required. The intensity of interfacial phenomena such as adhesion, friction and biocorrosion is proportional to the contact area [3].

Biocorrosion leads to the release of metallic ions from dental alloys [4-6]. Released metal cations may have adverse biological effects depending on the ion species and its

concentration [4]. Reports have shown that the amount of metal ions released from alloys with rougher surface is expected to be much higher than from alloys with smoother surface [5,6].

Cobalt-chromium (CoCr), a base metal alloy has been widely used in the fabrication of fixed (FPD) and removable partial dentures (RPD) since being introduced to dentistry in 1929. [7]. Cobalt-chromium alloy is also widely used in medicine as artificial hip replacement. Base alloys have increasingly replaced gold alloys owing to their improved mechanical properties and decreased cost [8-10]. The physico-chemical properties of base metal alloys include a lower density than gold alloys and a modulus of elasticity that is nearly twice that of gold alloys providing FPDs and RPDs with the advantage of maintaining rigidity with less bulk [10]. However, technical shortcomings include the increased difficulty of grinding and polishing procedures [11]. Due to the hardness of these alloys, special equipment is required for cleaning and smoothing the restoration after casting. In an *in vivo* study, Quirynen et al. [12] concluded that the increased surface roughness of RPD due to inadequate polishing procedures significantly increased the adhesion of bacterial plaque. Higher surface roughness of any material used in oral cavity is increasing the incidence of dental caries, gingivitis, and periodontal disease [13-16]. The release of metal ions has been reported not only from base metal alloys [4, 17], but also from high-noble and noble alloys [17-22]. The effect of finishing and polishing on surface roughness of cobalt-chromium castings revealed that smooth surfaces may improve oral health, decrease plaque retention, and increase alloy resistance to corrosion [23].

Biomedically acceptable Co-Cr dental alloys have been reported to have surfaces with dendritic microstructure produced during solidification forming dark interdendritic regions and light dentritic regions, as viewed with an optical microscope or SEM [24,25].

Different techniques have been used for measuring surface roughness, being the most usual nowadays AFM, SEM and optical profilometry [26]. The SEM has long been the standard means of investigation of surface morphology. Both, SEM and AFM techniques resolve structure down to the nanometer scale. However, the image formation mechanisms are quite different. SEM uses an electron beam operated under vacuum to give a two-dimensional 'photographic' image of the samples; but cannot directly provide quantitative data regarding the topography [27,28]. In contrast, the AFM technique reconstructs, in real time, the three-dimensional image of the sample topography and records the experimental data in digital form as sets of *z* (vertical axis), *x* (horizontal axis), and *y* (transversal axis) values [26-29].

In dentistry AFM has been used to evaluate tooth enamel surface [30-32], effects of etching and bleaching agents to tooth tissues [33-35], dentine surface characteristics and dentine-cementum junction characteristics [35-41]. Several studies were conducted to evaluate dental materials or instruments by means of AFM [42-47], such as stainless steel endodontic instruments [43], surface characteristics of NiTi and stainless steel orthodontic wires [40,45,47].

To our knowledge, there has been no well documented information about a 3-D characteristics of the surface of the cast CoCrMo dental alloy, or the effect of different polishing procedures on surface appearance and surface roughness, in particular. There are also no well documented information about the surface characteristics after exposure of CoCrMo dental alloy in solutions mimicking conditions in oral cavity, such as *saliva* and dentobacterial *plaque*. Therefore the aim of this study was to acquire qualitative and

quantitative nano-scale data of surface topography and surface roughness of CoCr dental alloy submitted to electropolishing and further to additional mechanical polishing procedures, following protocols which are generally used in dental laboratories. Another aim was to assess the surfaces of CoCrMo alloy after exposure into different solutions simulating oral conditions.

MATERIALS AND METHODS

Sample Casting

Eighteen CoCr alloy samples (Wironit® extra-hard, Bego, Germany), consisting of 63% Co, 30% Cr, 5% Mo, and Si, Mn and C < 2% were prepared and cast. Square shaped wax patterns 8x8x2 mm were prepared from a modelling pink wax (Modelling wax standard, DeguDent GmbH, Germany) and invested in the Castorit super C investment material for non-precious alloys (Dentaurum, Ispringen, Germany) according to the manufacturer's recommendation. Preheating was made at a temperature of 1000 °C and afterwards specimens were cast using a vacuum casting machine (Nautilus, Bego, Germany) at a temperature of 1420 °C according to the manufacturer's recommendation. The cast specimens were carefully cleaned from the particles of the investment material using a sandblasting machine (Austenal Dentastrahl VE2, Germany) with airborne-particles of aluminium oxide (250 µm) to remove investment residues.

Polishing Procedure

After sandblasting all the prepared samples were electropolished (Eltropol SL electropolishing machine, Bego, Germany) using an electrolyte polishing solution for CoCr alloys (Megalyt Megadental GmbH, Germany) for 15 minutes. The samples were divided into three groups, each group comprising six samples. The first group included only electropolished samples (EP). The second group included another six samples which were after electropolishing procedure, further mechanically polished using a rotating hard green rubber disk polisher (Fine-grit, Dentaurum, Ispringen, Germany) and afterwards using a high shine polishing paste (Sherapol 705, Shera GmbH, Germany) and a rotating polishing brush (black rotating polishing brush, radius 60 mm, Dentaurum, Germany) over a period of one minute. These samples were referred as black brush samples (BB). The third group comprised six samples, which were after EP procedure further mechanically polished using a rotating hard green rubber disk polisher (Fine-grit, Dentaurum, Ispringen, Germany) and afterwards using a high shine polishing paste (Sherapol 705, Shera GmbH, Germany) and a deer-leather rotating polishing wheel with a radius of 70 mm (Dlesk, Zagreb, Croatia) over a period of 3 minutes. These samples were referred as DL samples.

Sample Cleaning Prior to AFM Analysis

Prior to analysis, surfaces were carefully cleaned with alcohol, then rinsed with ultrapure water and finally sonicated for 10 min in ultrapure water. The samples were then dried by gentle blotting with absorbent paper, and stored in a plastic Petry dish.

AFM Analysis

Atomic force microscopy images of the CoCrMo samples were recorded using the contact mode operation on the Multimode AFM with Nanoscope IIIa controller (Veeco Instruments, Santa Barbara, CA, USA) under ambient conditions in air. The regions of interest (ROIs) were located using optical camera (Sony high-resolution CCD camera, Japan). V shaped cantilevers with pyramidal silicon-nitride tips (NP-20, Veeco), with nominal spring constant of 0.32 N/m were used. The scanner (JV) had maximum ranges of 125 μm in x- and y- direction and 5 μm in z- direction (height). The resolution was 512 x 512 pixels per image. The dimensions of scanned surface areas were: 13 x 13 μm^2 , 30 x 30 μm^2 and 100 x 100 μm^2 . AFM images were analysed off-line using the AFM NanoScope Control software, version 5.12r5 (Veeco Instruments, Santa Barbara, CA). All images presented are raw data except for the first order two-dimensional flattening. The roughness analysis acquired RMS values (root mean square average of height deviations taken from the mean plane); R_a values (arithmetic average of the absolute values of the surface height deviations measured from the mean plane) and Z-range values (maximum vertical distance between the highest and the lowest data points in the image). Mean values of five scanned surfaces 100 x 100 μm^2 of each specimen were accounted for statistical analysis. AFM analysis of EP samples was performed twice, the first time after EP procedure and the second time after being exposed in two different solutions (*saliva* and *plaque*) over the period of 30 days. To be able to locate the same ROIs prior and after soaking, the cross mark had been engraved, together with one dot in the upper left corner. Surface area of typical patterns of the CoCrMo images obtained by AFM were measured using ISSA software (VAMS, Zagreb, Croatia).

SEM Analysis

SEM images were recorded by detection of signals from the secondary electrons (SEI) and back scattered electrons (BSE) using Scanning Electron Microscope TESCAN VEGA LSH (Czech Republic) equipped with energy dispersive x-ray spectrometer (Oxfords' Instruments INCA system with software and detector technology for EDS microanalysis). These methods allow precise structural and elemental examination of surface details.

The surfaces of two EP samples were analysed prior and after immersion into *saliva* and *plaque* solutions for a period of 30 days.

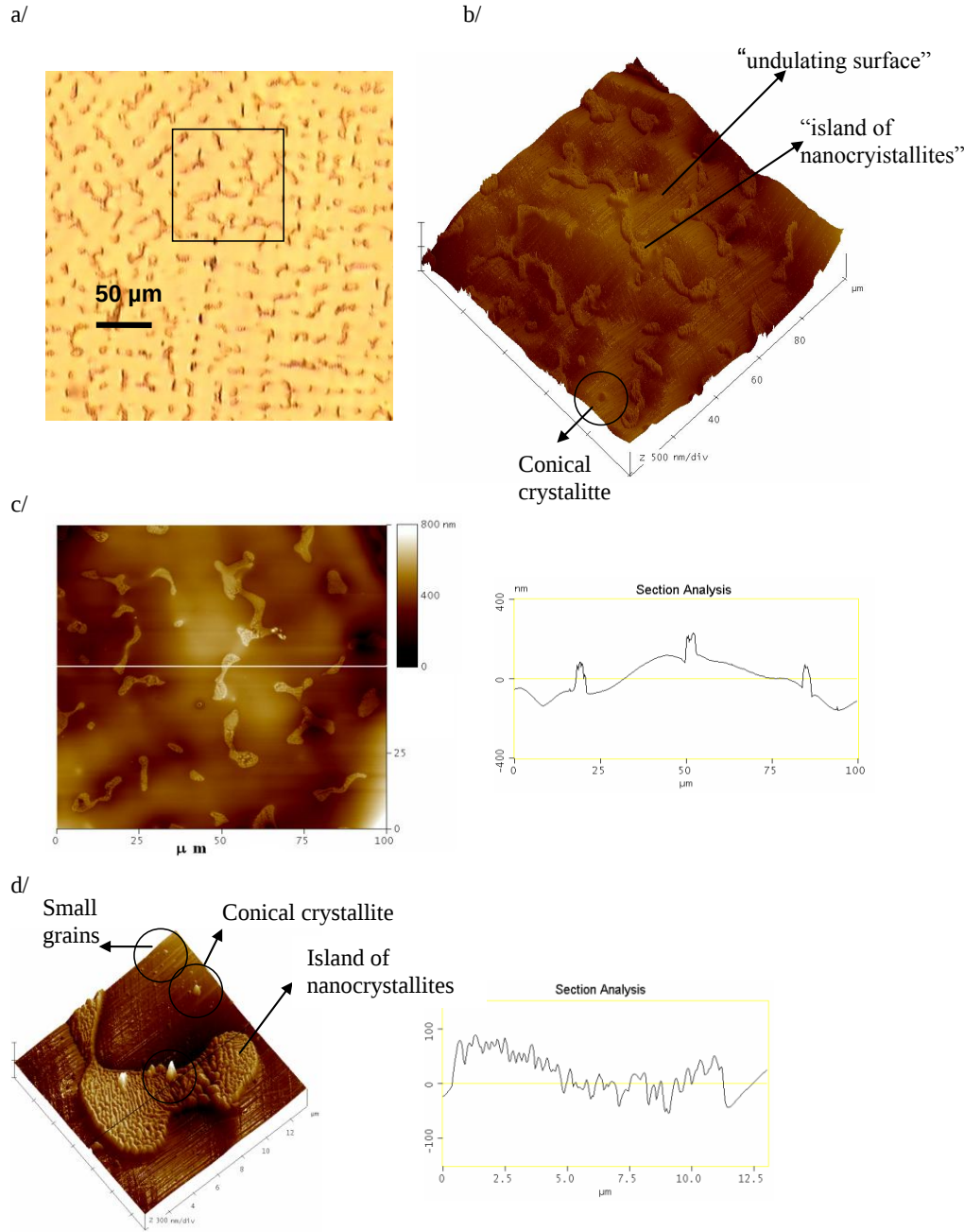


Figure 1. a/ The surface of electropolished (EP) CoCrMo alloy obtained by optical microscope (x 400); b/ 3-D AFM image of the surface marked with a box in a/, 100×100 μm (height 0.5μm); c/ AFM section analysis of the same surface as in b/.

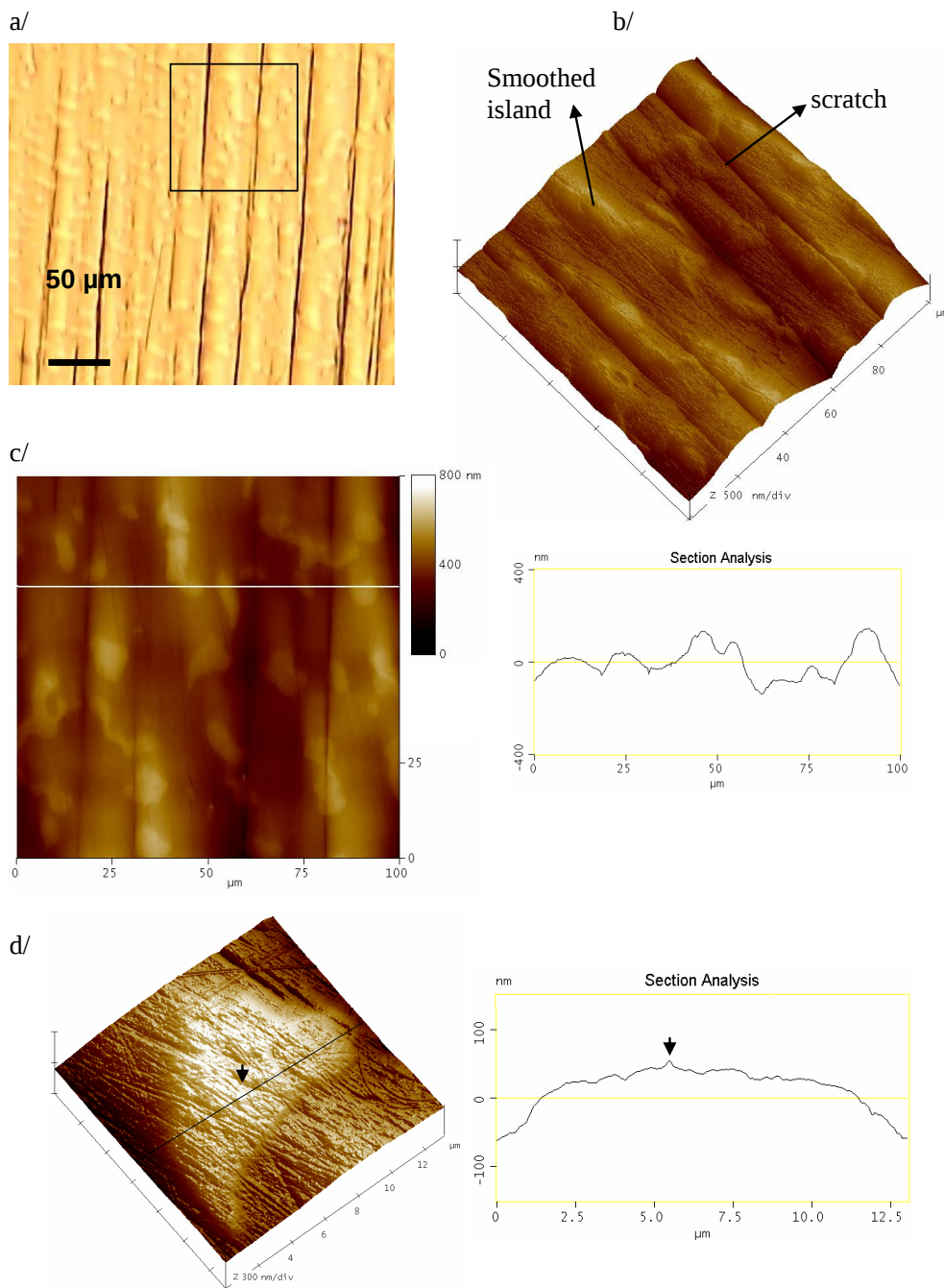


Figure 2. a/ The surface of mechanically polished (BB) surface of CoCrMo alloy obtained by optical microscope (x 400); b/ 3-D AFM image of the surface marked with a box in a/, 100x100 μm (height 0.5 μm); c/ AFM section analysis of the same surface as in b/ ; d/ 13x13 μm AFM 3-D surface shows one smoothed island, however one crystallite peak still remained (arrow).

Immersion (Extraction) Solutions

Two different buffered immersion solutions were prepared: (1) pH 6.0 phosphate buffer, to resemble pH of saliva (*Saliva*) and (3) pH 3.5 mixture of 0.1 M lactic acid, 0.1 M sodium chloride, 0.1% acetic acid, and 1.0% formic acid; (*pro analysis*, Kemika, Zagreb, Croatia) to resemble conditions under dentobacterial plaque (*Plaque*). Natural fresh saliva has a pH 5.7-7.0. Bacteria in the active dental plaque generate a powerful pH 4.0, or even lower, tooth mineral destroying mixture of lactic, formic, acetic and other metabolic acids [48].

Alloy Soaking

Individual 15 ml glass tubes with plastic stopper (culture tubes with GL thread, AR-Glass®, Brand GmbH, Wertheim, Germany) were used. Six replicates of EP CoCrMo alloys were immersed in 7 ml of *Saliva* and *Plaque* extraction solution, respectively, and incubated at 37 °C for 30 days.

Statistical Analysis

Statistical analysis was performed using the SPSS 14 for Windows (Chicago, Illinois, USA). One-way analysis of variance was used to test the differences between the three polishing procedures with Scheffe post hoc tests for multiple comparisons. The level of significance was set at $p < 0.05$.

RESULTS

Typical CoCrMo Surface

The surface of the electropolished CoCrMo sample is presented in Figure 1 (a-d). It is a typical surface topography of a sample after EP procedure. Figure 1a presents CoCrMo surface obtained by optical microscope (350x350 µm) with a pattern of light and dark regions. Figure 1 b presents a 3-D AFM image of the surface in a box (Figure a) (100x100 µm, height 500 nm). Islands of nanocrystalites emerging from the undulating surface represent typical formations of the surface. Undulations of the smooth surface are 20-40 µm long and 150-400 nm high (Figure 1c). The islands of crystallites have characteristic oblong shapes and the surface areas ranging from 4 to 80 µm². The islands' height (90-150 nm) can be determined precisely. Crystallites emerging in clusters are ordered in parallel rows 160 or 320 µm apart within the island. This periodicity is well documented in section analysis and better observed at higher resolution, as shown in Figure 1 d. The described surface topography is dominant surface pattern in all electropolished samples studied. Besides undulating surface and islands of nanocrystalites, some high conical crystalites (800-1000 nm) and small grains can also be observed, emerging from the undulated surface (Figure 1 b

and d). High conical crystallites were also emerging from the “islands” (Figure 1d). The surface area of “islands” varied from 9.8 to 18.6% of the surface.

Effect of Polishing

The surface of the mechanically polished Co/Cr/Mo/ sample using a black brush rotating wheel at the end of a mechanical polishing procedure (BB) is presented in Figure 2 (a-d). Effects of further mechanical polishing can be easily observed on the undulating surface, as well as on the islands of crystallites. Undulating surface now shows numerous scratches that appear in parallel fashion as a result of a mechanical polishing procedure (Figure 2 a-c). The most prominent scratches with V shaped profiles are 13-27 μm apart. Their surface openings are 0.5-2 μm wide. The scratches are 15-100 nm deep. The effect of mechanical polishing on the islands of crystallites is clearly observed, as the islands retain their contour (perimeter), but their roughness is dramatically decreased when observed at the nanoscale (Figure 2b-d). Clusters of crystallites can be no longer recognised, as they were mechanically smoothed down to the height of 40 nm. The optical micrograph (Figure 2 a) shows a surface area 312.5 x 312.5 μm with a pattern of lighter and darker surface with dark lines representing scratches. Lighter areas now correspond to the islands (dark regions in Figure 1a), as clusters of crystallites were smoothed (their top surface shows no saw-tooth pattern any more), while slightly darker areas correspond to the undulating surface (lighter regions in Figure 1a), having dark parallel scratches resulting from the BB polishing procedure.

Figure 3 shows typical topography of a sample which was, after EP procedure further mechanically polished using finally a deer leather rotating wheel (DL). The undulating surface has numerous scratches and their distances are smaller than in BB samples (Figure 2). In particular, contours of smoothed islands of crystallites are also altered by parallel scratches, best characterised by section analysis (Figure 3 d).

Due to the mechanical polishing (BB) peaks of crystallites have been taken away (Figure 2), however, one peak of a crystallite still remained as indicated by the arrow. In the DL sample (Figure 3) the mechanical polishing took away peaks of crystallites and caused scratches within the island as well; one crystallite still remained untouched. The peak identified by the arrow in section analysis corresponds to the remaining crystallite.

The mean values and standard deviations of Z-range, RMS and R_a values of samples undergone the three different polishing procedures are presented in the Table 1. The highest Z-range (maximum vertical distance between the highest and the lowest data points in the image) was obtained in the EP samples and Scheffe post hoc test revealed statistically significant difference in comparison with the BB and the DL samples ($p < 0.01$). The DL samples revealed higher Z-range values than the BB samples, but the difference was not statistically significant ($p > 0.05$). The highest RMS and R_a values were also obtained for the EP samples. The DL samples showed higher RMS and R_a values than the BB samples. The difference of RMS and R_a values was not statistically significant between the three different polishing procedures ($p > 0.05$). SEM analysis of one of EP samples (Figure 4 and Table 2) revealed that only the composition of the undulating surface approximately corresponded to the manufacturer's declared composition. The undulating surface was almost twice richer with Co than the islands (Table 2). The islands were richer in Cr, Mo and C.

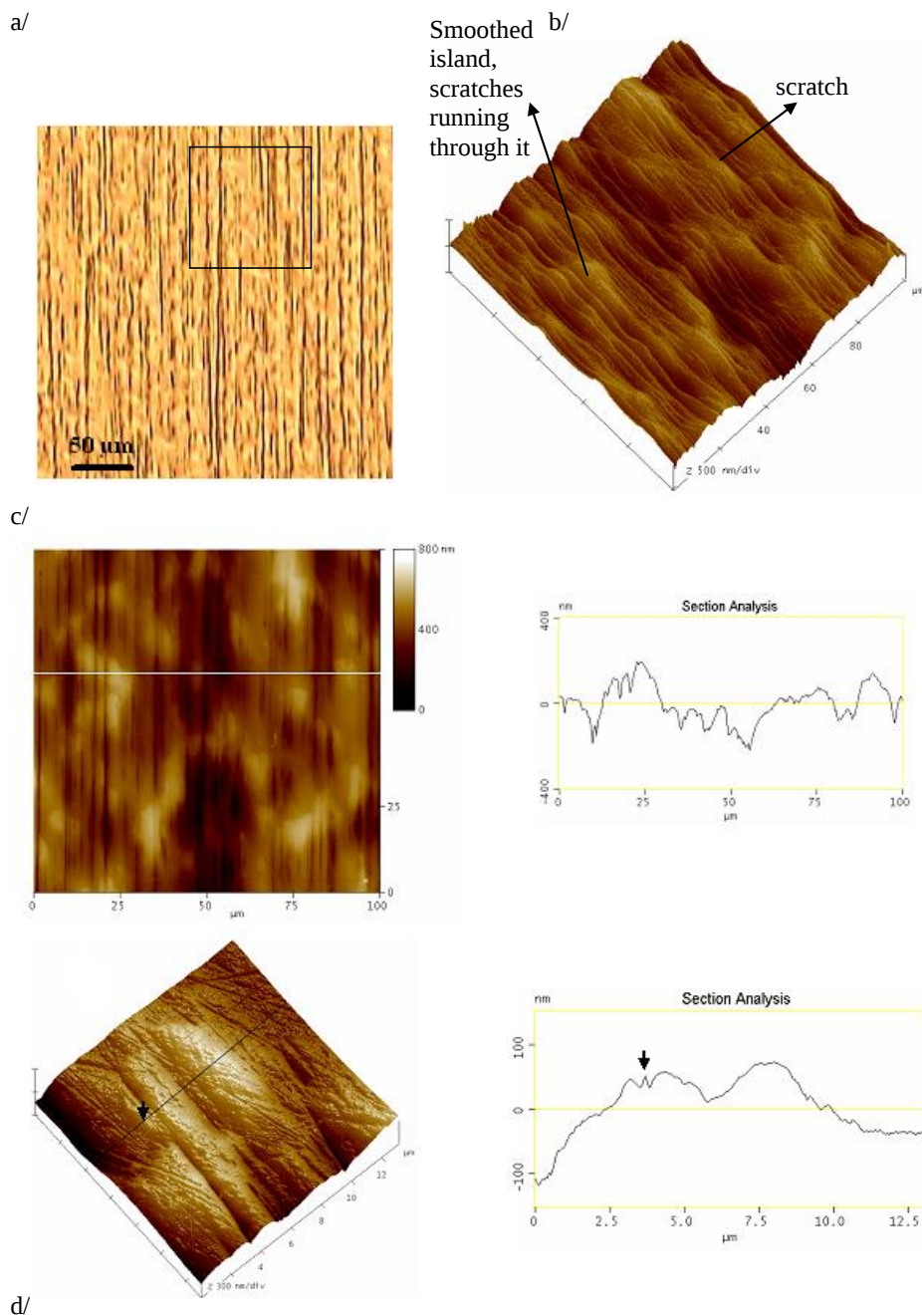


Figure 3. a/ The surface of mechanically polished (DL) surface of CoCrMo alloy obtained by optical microscope (x 400); b/ 3-D AFM image 100x100 μm (height 0.5 μm) of the surface marked with a box in Fig. a, c/ AFM section analysis of the same surface as in b/ ; d/ 13x13 μm AFM 3-D surface shows one smoothed island with a scratch running through it; section analysis clearly reveals that one crystallite peak still remained (arrow).

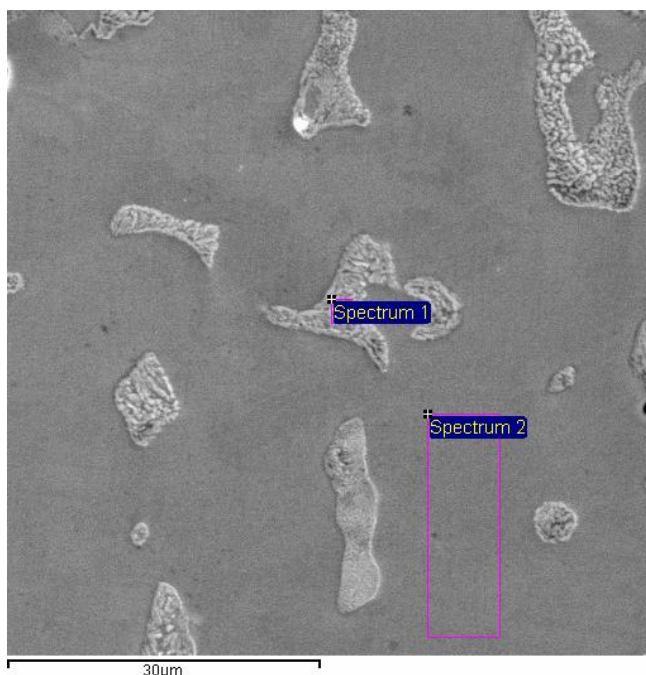


Figure 4. SEM with EDS probe: Spectrum 1=islands, Spectrum 2=undulating surface

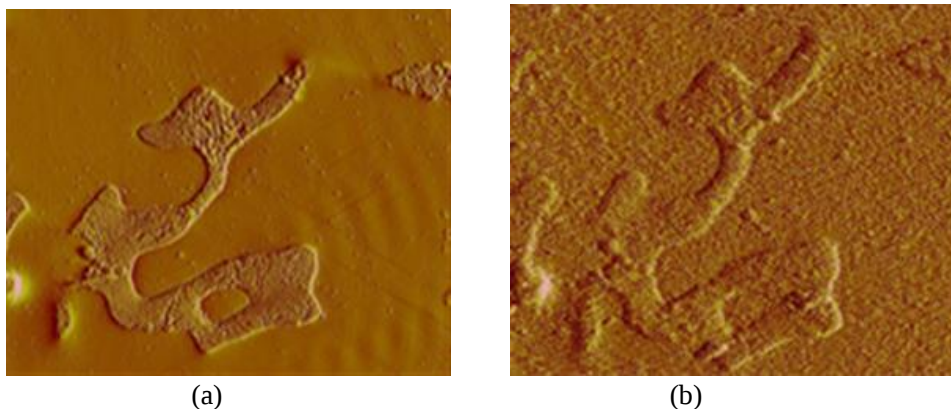


Figure 5. A surface of one EP sample (30x30 μm) before (a) and after (b) immersion in *saliva* for 30 days obtained by AFM.

Effect of Immersion in Saliva and Plaque Solutions

Figure 5 presents AFM image of the surface (30μm x 30μm) of one of the EP samples before and after immersion in *saliva* for 30 days. Figure 6 presents SEM image of one EP sample after immersion in *saliva* with position of EDS probe (spectrum). Peaks of main elements composing the alloy such as Co, Cr, Si, Mn, and C can be observed together with peaks of P, O, S, Na and Ti.

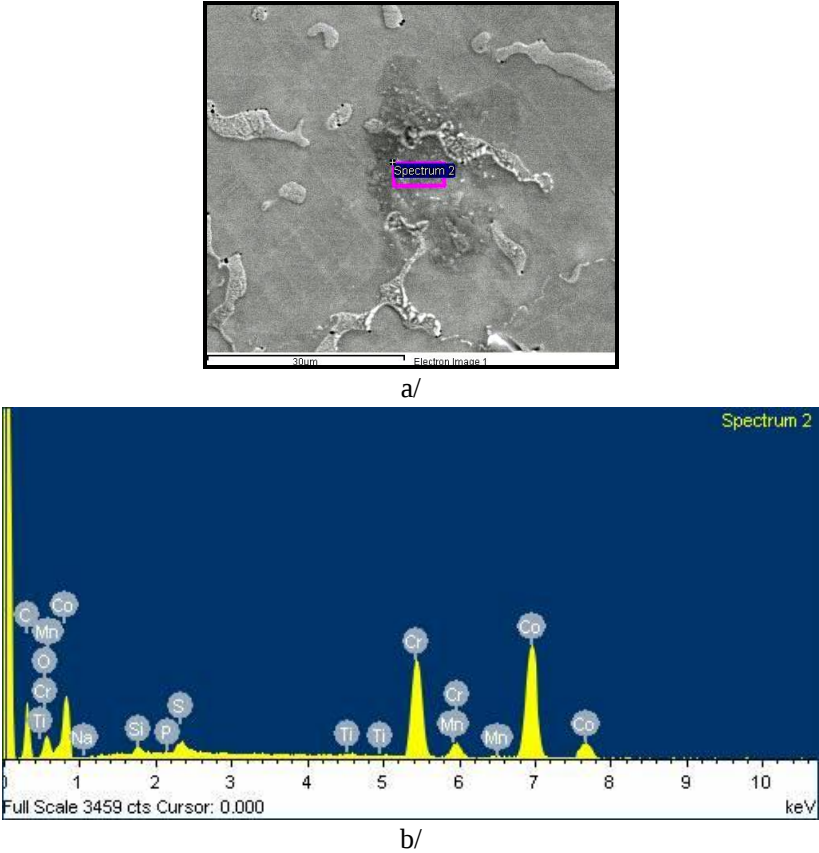


Figure 6. A sample after immersion in *saliva*: Position and EDS spectrum (a); Peaks of main elements composing the alloy such as Co, Cr, Si, Mn, and C, but also peaks of P, O, S and Ti (b).

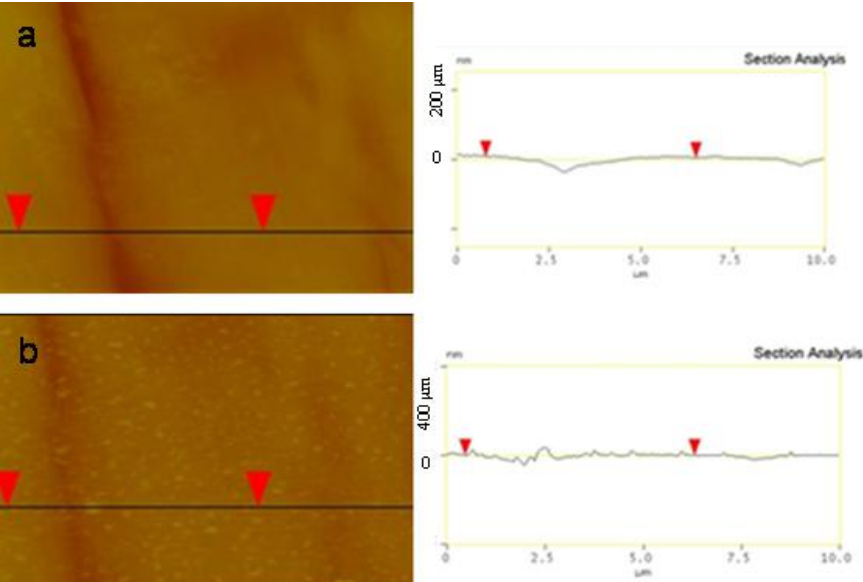


Figure 7. Section analysis of a surface before (a) and after (b) 30 days of immersion in *plaque*.

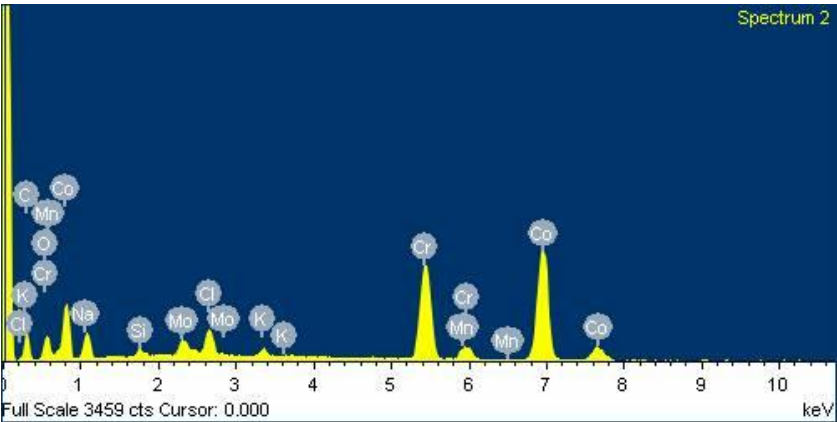


Figure 8. A sample after immersion in *plaque*: EDS-spectrum - peaks of main elements composing the alloy such as Co, Cr, Si, Mo, Mn, and C, but also peaks of K, O, Na and Cl.

Table 1. Surface roughness parameters obtained from AFM measurements: Mean values and standard deviations of Z range, RMS and Ra values from the surfaces of CoCr dental alloy after electropolishing (EP) and further mechanical polishing with black brush (BB) or deer leather wheel (DL)

Polishing procedure	Z-range (nm)	RMS (nm)	R _a (nm)
	x (SD)	x (SD)	x (SD)
Electropolishing (EP)	918.90 ^a (96.21) ^b	89.28 (19.49)	70.53 (14.79)
Black brush (BB)	537.23 (79.06)	77.97 (10.19)	61.72 (9.14)
Deer leather brush (DLB)	616.17 (58.72)	86.93 (13.48)	65.64 (9.72)

^a Statistically significant difference at $p<0.01$ (Scheffe post-hoc)

^b Standard deviations are given in parentheses

Table 2. Chemical composition (mass%) of the phases at the surface of Co/Cr/Mo alloy

Element	Co	Cr	Mo	Si	Mn	C	Total
Spectrum1	36.81	35.78	14.57	1.26	1.39	10. 20	100.00
Spectrum 2	62.37	26.14	4.66	0.70	1.31	4.83	100.00
Manufacturer’s declared composition	63	30	4	<2	<2	<2	100.0

Figure 7 presents AFM image together with section analysis of one of the samples before and after immersion in *plaque* for 30 days. Figure 8 presents SEM analysis: EDS peaks after immersion in *plaque*. Peaks of main elements composing the alloy such as Co, Cr, Si, Mo Mn, and C can be observed together with peaks of K, O, and Cl.

DISCUSSION

Surface topography and surface roughness of dental alloys are very important features as they determine the area of the contact surface with oral fluids and microorganisms in oral cavity.

The EP samples in the optical micrographs revealed a pattern of dark and light zones (Figure 1a). A similar pattern has been described previously [24,25] in CoCrMo cast alloys slowly cooled through the solidification range, as typical two-phase microstructure consisting of matrix (light) and dark regions. It was reported that light and dark regions correspond to the two types of CoCrMo solid solutions, which differed in structure (fcc vs. hcp), but only slightly in composition [24,25]. From the optical micrographs it is difficult to assess real topographic characteristics of the surfaces studied. In this study AFM images, together with topographic profiles (Figs. 1b-d) and roughness analysis provided actual topographic characteristics of the examined surfaces. The darker zones of EP samples in optical micrographs in Figure 1a can now, using AFM, be precisely identified as elevated islands, i.e. clusters of crystallites sharply emerging from the undulating surface (Figure 1 b-d). The crystallites within islands are 160 or 320 nm apart, which is best documented in section analysis (Figure 1c). Height of islands with only a few exceptions varied from 90-150 nm. The dark feature of the described solidification phase (crystallites forming islands) on optical micrographs is probably due to the reflectivity of the crystallite peaks within the islands as they have a saw-tooth pattern on the top (best seen in the section analysis, Figure 1 c and d).

Cast CoCrMo alloys are known to exhibit an inhomogeneous microstructure. The apparent two-phase microstructure seen in optical microscopy consists actually of many more phases. At higher magnification (SEM) interdendritic phase has been described to consist of blocky-shaped phase and lamellar structure, which further consists of at least two phases [49]. The CoCrMo cast alloy was described to consist of a Co-rich solid solution (dendrites) and an interdendritic four-phase mixture containing a cobalt-rich γ -phase, a chromium rich $M_{23}C_6$, an M_7C_6 phase and a chromium and molybdenum rich σ -phase [49]. There is however some disagreement as to whether interdendritic phases are in fact carbides, σ -phase, or a multi-phased region. Vander Sande et al. [50] identified interdendritic phase as σ -phase, while Zhuang and Langer thought that it consisted of carbides [51]. In the carbon free systems, in addition to the Co-solid solution, the σ -phase was identified, while carbides were identified in CoCrMo alloys containing carbon [49]. The results of this study reveal that alloy surface consists of islands of crystallites emerging from the undulating surface. However, AFM analysis found also some high conical crystalites and small grain-like crystallites at the surface (Figure 1d). High pointed conical crystalites can be found emerging both, the undulating surface and from the islands (Figure 1d). Chemical composition (mass%) of the phases at the surface of CoCrMo alloy clearly showed higher carbon content within islands of crystallites (Figure 4, Table 2), which indicates that this phase could consist of carbides. This study showed that the composition of undulating surface corresponded mostly to the manufacturer's declaration, except for the higher carbon content, which can be attributed to either pollution during manipulation, or the fact that the probe was in contact with the particular part of a surface with higher carbon content (eg. small grain-like crystallite, or similar).

Most base metal alloys, such as cobalt-chrome, are stable in the oral environment as a result of passive oxide layers that cover their surface. These oxide layers act as a corrosion barrier. In a Co/Cr alloy the oxide layer is only 1-4 nm thick [52].

After immersion in *Saliva* for 30 days many small grain-like formations can be seen at same parts or over the whole surface (Figs. 5 and 6), representing deposited particles. SEM with EDS showed peaks of main elements composing the alloy, such as Co, Cr, Si, Mn, and C, but it also showed peaks of P, O, S and Ti. Titanium is probably added to the alloy in a

very small quantities by manufacturer (not declared) and oxygen peak is probably from the Cr_2O_3 (oxide layer), which is only a few nanometers thick and prevents corrosion processes [52]. Peak of phosphorus was attributed to the phosphate anion from phosphate buffer of the immersion solution (*Saliva*, pH 6) and possible phosphate salt formation with the metals of the surface. Peak of sulfur was attributed to the anions from electrolyte polishing solution (Megalyt Megadental GmbH, Germany) which contains 10% sulphuric acid. We do not know whether salts were formed with the metals at the surface, or the metal ions were first released from the alloy and then precipitated, which is less likely (samples were thoroughly cleaned and sonicated).

After immersion in *Plaque* immersion solution, new small grain-like formation can also be seen clearly in Figure 7. Peaks of main elements composing the alloy such as Co, Cr, Si, Mo, Mn, and C can be observed. Moreover, peaks of K, O, and Cl also emerged. Oxygen peak is probably due to the Cr_2O_3 , while potassium, sodium and chlorine are probably from the buffer anions in *plaque*. It is not known whether new structures at the surface are chemically salts formed with surface metals, or precipitations, or something else, and it will be the subject of further study. We assume that small grains represent salts, and this further indicates that the layer of Cr_2O_3 was broken during immersion allowing salt formation.

We also studied the effect of mechanical polishing upon the EP surface. The effect of further mechanical polishing can be observed within the islands, as well as within the undulating surface. The most profound effect of further mechanical polishing is the change within the islands' morphology in the vertical direction (Figure 2 and 3). Due to the mechanical polishing (BB and DL samples) peaks of crystallites forming islands were taken away to a similar extent and their roughness was dramatically decreased when observed at the nanoscale. Therefore the surface within the islands became almost featureless and smooth (Figs. 2 and 3). The island's perimeter could still be precisely determined. Due to the fact that the islands' height was reduced and smoothed by mechanical polishing (peaks of crystallites were mechanically taken away and islands bear no more a saw-tooth pattern on the top), the darker regions observed in the optical micrographs of EP samples (Figure 1 a) now, after polishing turned to be lighter zones (Figs. 2 a and 3 a). Alteration from darker into lighter zones is attributed to the changes of reflectivity within islands (disappearance of the saw tooth pattern on the top). Another effect of polishing, probably due to a black-brush rotating wheel were numerous scratches formed at the surface. The scratches were ordered in a parallel fashion (BB and DL samples). The scratches appeared as dark lines in optical micrographs (Figs. 2a and 3a) and were denser in the DL samples than in the BB samples. Scratches were also observed within smoothed islands, more often in DL samples (Figure 2 and 3). The depth of the scratches varied from 15 to 100 nm.

The RMS, R_a and Z range values for the statistical analysis were obtained from the $100 \times 100 \mu\text{m}^2$ surface areas of samples. Previous AFM studies have shown that the roughness parameters are scale-dependent [26] implying that RMS values increase until the scanned size becomes larger than the main topographical features responsible for the observed roughness (such as periodicity), and that afterwards the RMS values remain constant, no matter of the increase of a scanned surface area. If a sample surface area studied is too small, it is likely that features on the surface that are larger or have a large periodicity will be overlooked. Smaller scan sizes might not reveal all surface morphology and could therefore show «false» lower values [26]. However, topography of some details can be more precisely visualised and

measured from smaller scan sizes. Detail analysis, including section analysis was actually done from 30 μm x 30 μm and 13 μm x 13 μm scans in this study.

The Z range data had significantly the highest values in the EP samples ($p < 0.01$, Table 1) in comparison with electropolishing and further mechanical polishing (BB or DL samples) (Table 1, Figs. 1-4). The RMS and the R_a values were also the highest in the EP samples, but the difference was not significant ($p > 0.05$) compared with the DL and the BB samples (Table 1). Although the mechanical polishing (the BB and the DL samples) took away the peaks of crystallite clusters (islands were smoothed) and therefore the islands' height decreased, the new formations - scratches appeared, which have increased surface roughness. Therefore the effect of islands' smoothening could not significantly contribute to the overall decrease of surface roughness. The RMS is the most frequently quoted parameter for the surface roughness analysis (representing standard deviation height of the surface around some mean values), but it does not take into account the distance between the features of the surface. For example, a surface with a few high-amplitude features may have the same RMS value as one with more low-lying features [53]. It is therefore important to calculate other parameters that measure peaks and valleys and profile shape and spacing of a surface, such as Z values. Surface morphology and surface roughness are the factors of considerable interest in biomedical materials, as they reveal which shape of a surface controls its interaction with biological components [54]. The roughness value is of interest for a specific cell-material interaction. The ability to adhere depends on a number of factors such as the degree of chemical interaction between the two components, the proximity, and the area of contact. Considering the dimensions of bacteria (0.5–1 μm), and yeasts (4–10 μm) [55,56], there are probably no significant distinctions in the material roughness they "feel" on the surfaces of the samples examined in this study, which were polished in different manners. However, further studies will be necessary and will be designed to assess if (and how) surface polishing influences microhardness and corrosion behaviour of any of the two solidification phases (the clusters of crystallites within islands and the smooth undulating surface) on the surface of the CoCr dental alloy.

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Chapter 11

INTRACELLULAR DELIVERY OF GOLD NANOPARTICLES: APPLICATIONS IN NANOMEDICINE

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ABSTRACT

Nanotechnology has enormous potential in biomedical research including disease diagnosis and therapy. Inorganic nanoparticles have been adopted for biomedical research due to their unique optical and physical properties. Compared to conventional materials, inorganic nanomaterials have several advantages such as simple preparative process and precise control over their shape, composition and size. A major aim of medicine is the early and precise diagnosis of clinical conditions, providing an efficient treatment without secondary effects. Nanoparticle-based technologies have demonstrated especially high potential for medical purposes, ranging from diagnosing diseases to providing novel therapies. Among the metal nanoparticles, gold nanoparticles have proven to be powerful tools in various nanomedicinal applications because of their photo-optical properties and biocompatibility. However, the existing nanoparticle-based technologies must overcome several challenges, including selective nanoparticle delivery, potential toxicity, imaging of nanoparticles and therapeutic efficacy. This chapter describes a brief overview of the recent research on delivery of gold nanoparticles inside the cells and their potential applications in nanomedicine.

INTRODUCTION

Targeted entry into cells is an important area of research in drug delivery and therapy [1-4]. Site-specific delivery of drugs and therapeutics is essential to reduce drug toxicity and

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increase therapeutic effects. To achieve efficient and specific cellular drug delivery, one strategy is to modify or conjugate drug with a ligand that can be efficiently taken up by target cells via receptor-mediated endocytosis [5].

The application of nanoparticles to nanomedicine research has tremendous potential in biomedical research, disease diagnosis and therapy. Nanoparticles has drawn attraction because of their interesting surface properties. Most of the applications are based on the optical properties of nanoparticles such as surface Plasmon resonance, surface enhanced Raman scattering and strong photoluminescence. Among the metal nanoparticles gold nanoparticles have been used with a long history as a contrast agent for scanning electron and light microscopy in histopathology study [6]. Some complete review articles have recently been published on the biological applications of metal nanoparticles. [7, 8]. Cellular internalization of nanoparticles that have optical properties is very important in the cellular imaging and targeted therapy. However, intracellular delivery of metal nanoparticles is a challenging task as the cell membrane is a formidable barrier to foreign materials [9-11]. The surface modification of nanoparticles is important to overcome this limitation. Surface modifications of metal nanoparticles with biomolecules (peptides, aptamers, antibodies, and other molecules) has facilitated specific interactions of nanoparticles with target cells or organelles [12-16].

Colloidal gold nanoparticles are one of the attractive platforms for cancer diagnosis and therapy. Gold nanoparticles are attractive because it has been approved and used for treatment of human disease (e.g. rheumatoid arthritis) [17]. Gold nanoparticles are also attractive because they are relatively easy to synthesize. For efficient delivery of AuNPs into a living system it needs to overcome natural biological barriers such as the cell membrane and the reticuloendothelial system (RES). Gold makes an excellent intracellular targeting vector for three reasons. First, gold can be synthesized routinely in sizes varying continuously from 0.8 to 200 nm with <10% size dispersity. Second, gold can be modified with a large collection of small molecules, peptides, proteins, DNA, and polymers. Moreover, all of these functional elements can be combined on a single particle simultaneously, often via simple one-pot procedures. Finally, gold particles have enormous visible light extinction characteristics.

Synthesis of AuNPs

Several methods have been reported in the literature for the synthesis of AuNPs. The most common method is by chemical reduction of gold salts such as hydrogen tetrachlorate (HAuCl_4) using citrate [18] or borohydride [19, 20] as the reducing agent. To achieve better monodispersity Brown and Natan [21] have reported the synthesis of monodisperse AuNPs with diameters between 30 and 100 nm using a seeding approach. The method is based on the use of the surface of the AuNPs as a catalyst for the reduction of Au^{3+} by hydroxylamine. Later this method have been used to control the shape and size of the nanoparticles [22].

There are also several physical methods reported for the synthesis of AuNPs such as microwave irradiation [23-25], sonochemical method [26-29], UV irradiation [30-32], laser ablation [33, 34], thermolysis [35, 36], radicals induced method [37-39], and photochemical method [40].

Biological systems, masters of ambient condition chemistry, synthesize inorganic materials that are hierarchically organized from the nano- to the macroscale. Recent studies

on the use of microorganisms in the synthesis of nanoparticles are a relatively new and exciting area of research with considerable potential for development [41]. Intracellular and extracellular gold nanoparticles can be synthesized using fungi [42, 43].

Protein Mediated Delivery

Several groups have shown that large AuNPs are quickly opsonized by blood and eliminated by the reticulo endothelial system (RES) in mammalian cells [44-47]. Several research groups have grafted different biological macromolecules such as proteins, peptides or small molecules onto AuNPs to attempt cellular selectivity, internalization and localization within heterogeneous population of cancer cells in solid tumors. Proteins such as tumor necrosis factor α (TNF α) and anti-EGFR antibodies have been successfully grafted onto AuNPs surface and utilized in conjugation with hyperthermia to selectively kill cancer cells. Tumor necrosis factor α (TNF α) is a potent cytokine with anticancer efficacy. Additionally, TNF α is overexpressed in solid tumors. TNF α grafted onto AuNPs surface has reduced systemic toxicity compared to the native unconjugated TNF α . More importantly, the TNF α -AuNPs conjugate are able to accumulate preferentially in the tumor vasculature. Recently, one chemotherapy agent, Aurimune has been developed by CytImmune (Rockville, MD, USA) based on this technology. Aurimune or CYT-6091 is a multivalent drug that is assembled on 26-nm particles of colloidal gold and designed to deliver recombinant human TNF within solid tumors. The drug is manufactured by covalently linking molecules of TNF and thiol-derivatized polyethylene glycol (PEG-THIOL or PT) onto the surface of the colloidal gold nanoparticles [48]. PEG is used to avoid RES. They have shown that the drug is internalized into MC-38 tumor cells through TNF α -mediated RME. In combination with hyperthermia this multivalent drug is able to kill tumor cells via vascular damage. The survival of SCK mammary carcinoma cells assessed using *in vivo*/*in vitro* cell survival assay following treatment with PT-Au- TNF α and hyperthermia alone or combined. The combination treatment of SCK tumors *in vivo* reduced the *in vivo/in vitro* tumor cell survival to 0.05% immediately following heating at 42.5 C and to 0.005% at 18 hrs after heating. [49]. Most probably, this combined effect cause shutdown of tumor microvasculature and microvasculature and thus depriving the tumor microenvironment of nutrients and oxygen [50, 51]. Apparently, the microvascular shutdown makes the tumor tissue more susceptible to thermal injury, and large numbers of tumor cells were cut off from the circulation causing massive cell death and tumor regression.

Another receptor that can be targeted in therapy is epidermal growth factor receptor (EGFR) which is overexpressed in several cancers [52-57]. Monoclonal antibodies such as anti-EGFR antibody have been investigated as a possible anticancer therapy for lung cancer. AuNPs have been successfully conjugated with anti-EGFR antibody and employed as a cancer diagnostic tool [58] and for photothermal therapy [59, 60] in an oral cancer model. The color scattering property of the AuNPs has been used in designing anti-EGFR-AuNPs conjugates for diagnosis of HSC and HOC oral cancer. These conjugates bind to cancerous and noncancerous cells in a different manner. In case of HOC and HSC oral cancer cells where EGFR is overexpressed, these conjugates bind in a homogenous manner, whereas HaCaT, a noncancerous cell line in which EGFR expression is depressed, only showed a random AuNPs conjugate binding. Such binding preferences and unique light scattering

property of these conjugates provide a useful diagnostic tool to distinguish between noncancerous and cancerous cells. This concept has been successfully employed in imaging EGFR-enriched Tu696 human head-and-neck carcinoma cells *in vivo and in vitro* using an ScFr version of the anti-EGFR antibody [61].

Transferrin (TF) is also a suitable ligand for conjugating drugs, since it can be specifically recognized and taken up by TF receptors actively expressed on the surface of various tumors cells [62]. AuNPs-TF conjugation has been made to study the cellular uptake [63]. AuNPs are covalently coupled to TF molecules by mercaptoacetic acid. When the AuNPs-TF conjugates are incubated with human nasopharyngeal carcinoma cells (NPC, SUNE1), nanoparticles internalization into cells is visualized by a multimode Atomic Force Microscopy (AFM). The nanoparticle internalization into NPC cells is validated by using confocal laser scanning microscope (Figure 1). It has been demonstrated that endocytosis of TF-conjugated nanoparticles taking place during the process of internalization. Li et al have recently extended this idea in cancer cell imaging and therapy [64]. Gold nanoparticles are conjugated with transferrin molecules (Tf) for targeting, imaging and therapy of breast cancer cells (Hs578T). They have shown that, the transferrin-transferrin receptor-mediated cellular uptake of gold nanoparticles is six times of that in the absence of this interaction. To demonstrate the efficiency of the conjugates in selectively targeting cancer cells, they have also investigated normal cells. The cellular uptake of the conjugates by the normal cells (3T3) is only one fourth of that by cancerous cells. It is observed that dark field light scattering from cancer cells was enhanced when the cells are incubated with PEG-Tf-AuNPs and very weak scattering without nanoparticles. Photothermal therapy is carried out for cancer cells. It is shown that the cells without AuNPs could not be killed by laser even at a very high power density of 114 W/cm^2 due to the weak light absorption of the cells. However, when the cells are incubated with PEG-AuNPs and PEG-Tf-AuNPs, significant damage to the cells is observed at 7 W/cm^2 in presence of later conjugates. This could be due to the presence of more gold nanoparticles inside cells as a result of specific transferring receptor-mediated cellular uptake, as suggested by the authors. The result demonstrates that this conjugate can be potentially used for cancer diagnostics and therapy.

Peptide Mediated Delivery

Peptides are also good vehicles for AuNP delivery. Most peptides used for AuNPs delivery target the cell nucleus. The nucleus is an important target for photothermal therapy because it contains the cellular genetic machinery. These nuclear membrane-penetrating peptides facilitate the entry of AuNPs into the nuclei by nuclear localization through interaction with the nuclear pore complex [65]. Most of the nuclear membrane-penetrating peptides are derived from virus sources, such as Simian virus [65-67], adenovirus [65], HIV1 [68]. Some RGD peptides have been used to deliver AuNPs [69, 70]. Targeted nuclear delivery is a challenging task, however, as any cell-specific nuclear probe must satisfy the following requirements: it must (i) be small enough to enter cells and cross the nuclear membrane ($<100 \text{ nm}$ for uptake by receptor-mediated endocytosis (RME) and $<30 \text{ nm}$ for import through nuclear pores), (ii) penetrate cellular membranes or bind to cell-specific plasma membrane receptors, (iii) bypass or escape endosomal/lysosomal pathways, (iv)

penetrate nuclear membranes or access importins to pass through the nuclear pore complex, and (v) have low toxicity.

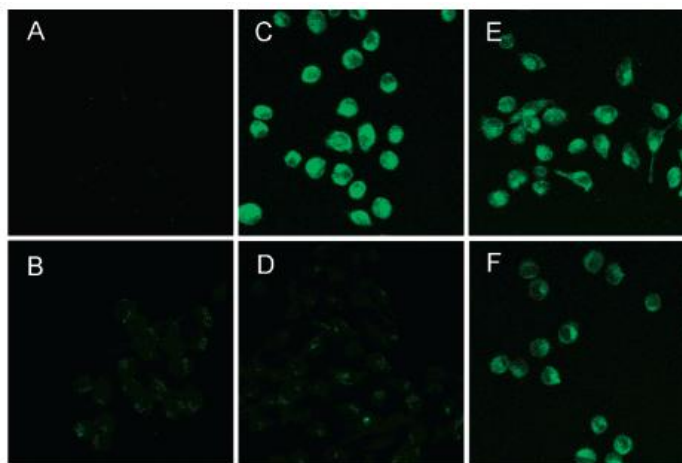


Figure 1. Confocal cell images show that Au-TF nanoparticles were internalized into the cellular plasma of NPC cells via Tf receptor interaction pathway (5-h treatment). (A) Control cells without Au-TF nanoparticles; (B) cells treated with Au nanoparticles alone; (C) cells incubating with Au-TF nanoparticles; (D) cells treated with Au-albumin nanoparticles; (E and F) cells cotreated with different proportions of Au-TF versus holo-TF (1:2 and 1:5, respectively). Reprinted with permission from ref (63). Copyright 2005, American Chemical Society.

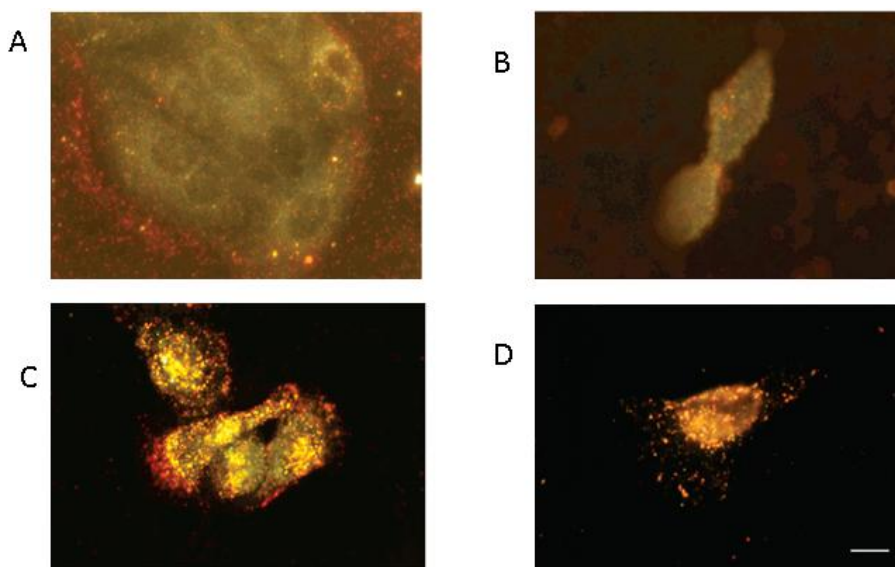


Figure 2. Dark field light scattering images of cells after incubation with gold nanorods and peptide-conjugated gold nanorods for 2 h. A. HaCat normal cells incubated with gold nanorods. B. HSC cancer cells incubated with gold nanorods. C. HaCat normal cells incubated with peptide-conjugated gold nanorods. D. HSC cancer cells incubated with peptide-conjugated gold nanorods. Peptide conjugation promotes the cellular uptake of gold nanorods. Scale bar: 10 μm . Reprinted with permission from ref (67). Copyright 2007, American Chemical Society.

Among the nuclear penetrating peptides identified so far, simian virus nuclear localization peptides (NLS) is very well-known to target nucleus. Several groups have used NLS peptides to deliver AuNPs inside cells [65, 69, 71]. NLS peptides successfully facilitate the entrance of the conjugate into the nucleus of HepG2 when directly injected inside the cytoplasm [65, 72]. However, the conjugates are trapped inside the cytoplasm when included in the cell growth media. Feldherr et al have extended their study by using different size of gold nanoparticles in simian virus 40 (SV 40) transformed cells [73]. Transformation is dependent on the expression of the early region of the SV40 genome that encodes the large (T) and small (t) tumor antigen [74, 75]. T antigen is a multifunctional protein that contains a NLS. The experimental cells are microinjected with nucleoplasmin-coated gold particles that ranged in diameter from 110 to 320 Å, including the 30 Å protein coat. Nucleoplasmin is a karyophilic *Xenopus oocyte* protein that contains a well characterized NLS [76] and can effectively target gold particles to the nucleus [77]. It is observed that nuclear uptake was significantly higher in the transformed cells. In addition, measurements of particle size reveals a significantly increased nuclear accumulation of gold particles in the 200 to 240 Å size class. Particles larger than 280 Å (or 310 Å including protein coat) are unable to enter the nuclei. These results demonstrate that the exclusion limit for particles entering the nucleus was 40 Å greater in transformed cells compared with nontransformed cells. In these studies the nanoparticle-peptide complex was injected directly into the cytoplasm near the nucleus, thus bypassing cellular membrane entry and endosomal /lysosomal pathways. Later Tkachenko et al have investigated on the basis of this established procedure to examine the ability of a number of viral targeting peptides to target the nucleus from outside intact cells [69]. Gold-peptide conjugates are evaluated for their subcellular distribution in HeLa human cervical epithelium cells, 3T3/NIH murine fibroblastoma cells, and HepG2 human hepatocarcinoma cells. Video-enhanced color differential interference contrast microscopy and transmission electron microscopy indicates that transport of nanoparticles into the cytoplasm and nucleus depends on peptide sequence and cell line. In case of HeLa cells, peptide-nanoparticle conjugates are found clustered together in cytoplasm and also few located inside the nucleus. None of the conjugates are able to translocate the nuclear membrane in 3T3/NIH cells. Entry into the cytoplasm of HepG2 cells is strongly dependent on the peptide sequence attached to nanoparticles. The conjugates are able to enter the cytoplasm but were not found near the nuclear membrane or inside the nucleus, which suggests that these conjugates are unable to escape from endosomes. The SV40 NLS is capable of entering HeLa cells via receptor-mediated endocytosis. It is also able to cross the nuclear membrane if it is directly injected into the cytoplasm [73]. Regardless of this, the SV40 NLS is not translocated from outside an intact HeLa cell to the nucleus because it cannot break free of endosomal compartments or is somehow altered as it travels through the endosomal pathway. Gold nanospheres used in these conjugates only absorb in the visible region, thus limiting their use in *in vitro* applications such as in photothermal therapy, which requires near-infrared region. To address this issue, Oyeler et al have used gold nanorods and made AuNPs-NLS peptide conjugates through a thioalkyl-triazole linker [67]. Cellular uptake of CTAB-capped gold nanorods and thioalkyl-triazole-peptide conjugated gold nanorods has been studied. Peptide conjugated gold nanorods have higher efficiency compared with CTAB-capped gold nanorods (Figure 2). This enhanced gold nanorod uptake may be due to the receptor-mediated endocytosis. Moreover, it is found that the thioalkyl-triazole-peptide conjugated nanorods are distributed into both nucleus and cytoplasm in either

normal or cancer cells. However, the nanorods are more concentrated in the nucleus of the cancer cells. This may be due to the increased specific and active uptake of the NLS peptide or decreased regulation of passive uptake of cytoplasmic components due to the disruption of the normal cellular process. This group also discovers that Raman signal distinguishes the malignant from nonmalignant cells. This work demonstrates that gold nanorods can be used for nuclear targeting as a new type of imaging-based contrast agents.

Peptides derived from adenovirus have also been studied to promote nuclear penetration [65, 69]. The protein sequence from adenovirus contains both RME and NLS domains [65]. The AuNPs conjugates of this peptide has been shown to successfully avoid the endosomes and penetrates the nucleus of the HepG2 cells [65, 69]. The ability of individual RME and NLS domains derived from the adenovirus protein has been investigated separately for nuclear penetration. Adenoviral RME only able to deliver AuNPs into cytoplasm. NLS sequence of adenovirus protein is unable to enter into HepG2 cells [65], however it delivers AuNPs into the cytoplasm of 3T3/NIH cells and some nuclear translocation has been observed in HeLa cells [69]. Nonetheless, combination of adenoviral RME and NLS conjugated to AuNPs is able to penetrate the nucleus of HepG2 cells. These conjugates show preferential nuclear entry in comparison to the adenovirus protein that contains both RME and NLS sequence [65, 78].

Another nuclear penetrating peptide is derived from HIV1 Tat-protein. This peptide sequence has been used as an efficient way of internalizing a number of marker proteins in cells [79, 80]. Gold nanoparticles are conjugated with Tat peptide via a non-amino acid linker, tiopronin [68]. These nanoparticles are biocompatible. Both Au-tiopronin and Au-Tat conjugates have been studied for cellular uptake. These conjugates are taken up by the primary human fibroblasts cells (HTERT-BJ1). While Au-Tat particles are mainly located in the nucleus, only a few Au-tiopronin particles appear to be taken into the cell through membrane invaginations and accumulate in the cytoplasm in vacuoles or surrounding the mitochondria (Figure 3). Au-tiopronin nanoparticles are not detected in the nucleus in any case. This demonstrates the specificity of Au-Tat particles to be translocated and accumulate in the nucleus. This research has many potential applications in cell imaging, but also extends wider biomedical applications, such as gene therapy and drug delivery.

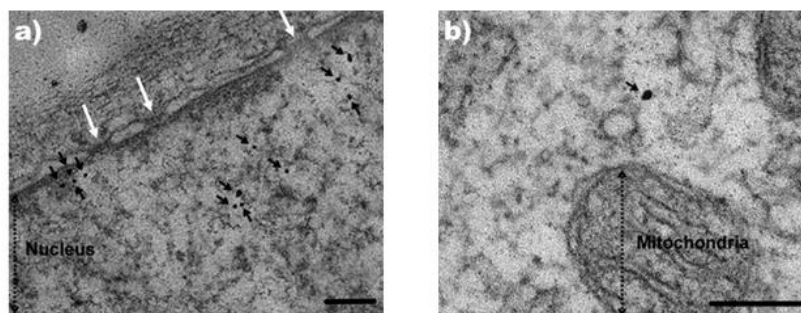


Figure 3. Transmission electron micrographs of human fibroblasts incubated with Au@Tat (a) and Au@tiopronin (b) nanoparticles. The black dots indicated with arrows are nanoparticles, and white arrows show nuclear membrane pores. (scale bars) 50 nm). Reprinted with permission from ref (68). Copyright 2005, American Chemical Society.

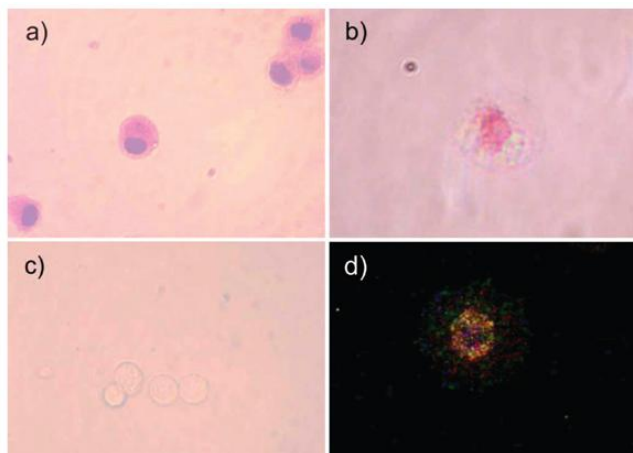


Figure 4. Bright-field microscopic images of HeLa cell after being incubated with 0.04 nM 30 nm gold nanoparticle-peptide complexes for 24 h: (a) mixed peptides (CALNNR8/CALNN) 1:9), (b) pure CALNNR8, and (c) pure CALNN-functionalized gold nanoparticles. (d) Corresponding dark-field microscopic image of one cell in (a). Reprinted with permission from ref (83). Copyright 2008, American Chemical Society.

Peptide sequence RGD is also used for intracellular delivery of gold nanoparticles. But these peptides do not induce nuclear translocation. RGD receptors are overexpressed on the surface of human fibroblast cells. But it is relevant to mention that RGD peptide should be linked through an appropriate linking moiety to the AuNPs to avoid loss of RGD-mediated RME. AuNPs-tiopronin conjugates can be linked to RGD peptide through secondary linkers, such as ethylenediamine (EDA) and poly(ethylene glycol) bis (3-aminopropyl) terminated (PEG) [81]. As expected for a RME-sequenced peptide, RGD-EDA-tiopronin-AuNPs conjugates are internalized within the cytoplasm of the human fibroblast cells. However, in case of RGD-PEG-tiopronin-AuNPs conjugates no internalization is observed [70].

Though RGD-derived peptides are unable to enter nucleus, recently Tkachenko et al has discovered that modification of these peptides are capable of targeting the nucleus of HepG2 cells and HeLa cells, when conjugated with AuNPs. These modified peptides are composed of the integrin binding domain (RGD) and a stretch of six lysine residues [69]. Integrin binding domains are known to induce cellular uptake by both RME and phagocytosis. However, usually they do not interact with the nuclear pore complex. So, probably these conjugates enter the cell via the integrin binding domain and escape endosomes due to the presence of positively charged lysines, which are known to induce leakage of negatively charged vesicles and cells.

Recently, cell penetrating peptides have been used to deliver AuNPs into osteosarcoma (bone cancer) cells [82]. Here, we modified the peptides with cysteine terminus to attach with gold nanoparticles. Three types of peptides have been studied. Cell penetrating peptides (CPPs) and NLS attached gold nanoparticles efficiently enter inside cells. But exceptionally high uptake of conjugates by cells is observed when CPPs coupled with NLS. After 3h of incubation with the cells, most of the internalized peptide-nanoparticles conjugates are found clustered together inside membrane -bound cellular compartments that appear to be endosomes as observed by TEM.

Arginine-rich peptide (CALNNR₈)-functionalized gold nanoparticles have been prepared for the purpose of cellular membrane transportation where CALNN is used as a stabilizing agent [83]. The AuNPs-peptide complexes exhibit specific recognition properties for nucleus and endoplasmic reticulum targeting. The translocation ability of AuNPs-peptide complexes is dependent on the ratio of CALNNR₈ to CALNN on the particle surface and the nanoparticle diameter. Mixed peptide (CALNN and CALNNR₈)-functionalized AuNPs are capable of targeting the cell nucleus. However, AuNPs-peptide complexes synthesized by pure CALNNR₈ do not reach the nucleus (Figure 4). It indicates that not only the arginine part of the carrier peptides direct the internalized localization, but also the stabilizing peptide, CALNN, contributes to this process.

Very recently, Pujals et al has shown that AuNPs can be shuttled into tumoral cells with an amphipathic proline-rich peptides, (VRLPPP)₃ which is also known as sweet arrow peptide (SAP) [84].

Small Molecule Mediated Delivery

AuNPs can be delivered by small molecule therapeutic agents into tumor. Upon accumulation at the tumor site, the attached small molecule facilitates a RME mediated uptake of the AuNPs into the diseased cells. One example of such small molecule is folic acid (FA), a natural B-vitamine has been exploited to selectively target folate receptor (FR) expressing cells [85-89]. FR is a useful target for tumor-specific drug delivery because this is highly expressed on epithelial cancers of the ovary, brain, throat kidney, colon, lung, prostate, breast and myeloid cells etc. [86]. Conjugating FA and a therapeutic agent to metal nanoparticles is an important step for FR-mediated targeted drug delivery to cancer cells. AuNPs-folate conjugates show promising result in selective targeting of FR-positive tumors. These conjugates have been used in tumor imaging and photothermal therapy applications [90]. A poly(ethylene glycol) (PEG) construct with thioctic acid and folic acid coupled on opposite ends of the polymer chain has been synthesized with AuNPs for targeting the FR expressing tumor cells via RME. The resulting coated AuNPs are water soluble and are designed as (i) mPEG-thioctic acid-modified AuNPs (mPEG 2000 – T:AuNP) or (ii) the folate-PEG 1500- thioctic acid- modified AuNPs (F-PEG1500-T: AuNP). Internalization of these conjugates have been studied in KB cells (human epithelial carcinoma cell line that expresses the FR) and WI-38 cells (a normal embryonic human diploid cell line as a folate receptor deficient cells). Efficient internalization has been observed in case of tumor cells expressing the FRs. AuNPs-conjugates uptake is minimal in other types of cancer cells that lack FRs.

There are only few reports that describe the attachment of FA to metal-based nanoparticles using covalent interaction [90, 91]. Recently, a simple method of attaching FA to AuNPs has been demonstrated using noncovalent interaction [92]. Different PEG backbones has been used to tune the attachment process. Successful delivery has been achieved to different cancer cells (SKOV-3, OVCAR-5, OV-202, OV-167, OPM-1, RPMI and U266) with differential FR expression. Quantitation of the internalization of the nanoconjugates in different cell lines is determined by gold analysis with inductively Coupled plasma. Uptake of the nanoconjugates is correlated with FR expression. TEM imaging of treated cells reveals the endocytosis of the nanoconjugates. Dendromer-entrapped, folate functionalized AuNPs have

also shown the potential for targeting and imaging cancer cells [93]. when KB cells treated with this conjugate selective uptake by the high FR-expressing cells has been observed. Methotrexate (MTX), an analogue of folic acid, when conjugated to AuNPs avoid tumor cell resistance which invariably developed upon repeated use of this flexible anticancer drug [94]. It is observed that MTX-AuNPs conjugates rapidly accumulated in LL2 (Lewis lung carcinoma) cells, inducing higher cytotoxic effects on the tumor compared with free MTX which shows no antitumor effects.

AuNPs conjugates of other chemotherapeutic agents have been reported to address various limitations of the unconjugated agents [95, 96]. A multifunctional vector consisting of AuNPs, TNF α , thiolated PEG and paclitaxel (PTX), a leading anticancer drug has been reported for targeted drug delivery to solid tumors [47]. The release of PTX from the vector is investigated in B16/F10 melanoma cancer cells. It is observed that the vector remains inactive unless treated with dithiothreitol (DTT). It indicates that the vector is acting as a prodrug from which PTX must be released to show the desired anticancer effects. It is shown that PTX-PT-AuNPs-TNF α vector delivers 10-fold more TNF α and PTX to the tumor site when compared to unconjugated ones. Recently, Mukherjee et al has developed similar type of vector consisting of two components with different functions: an antiangiogenic molecule, VEGF antibody-2C3 (AbVF), and an anticancer drug, gemcitabine [97]. They have developed this '2 in 1' system onto a single AuNP core. AuNPs conjugates of fluorescent small molecule, such as coumarin, have been developed as cellular probes and delivery agents [98]. Attachment of PEG-functionalized AuNPs to coumarin through carbamate bond shows significant enhancement of emission intensity. AuNPs conjugates are efficiently internalized in MDA-MB-231 cells and localized in the perinuclear region as evidenced through intracellular particle tracking. Very recently, Kahalalide F, an antitumoral drug has been conjugated with gold nanoparticles [99]. The conjugation of AuNPs to an antitumoral drug could play two parallel roles: to enhance the delivery of a drug to the cell by presenting numerous peptide copies anchored to the nanoparticle surface and to help in the delivery of the nanoparticle to the pathological cell favoring the local aggregation of nanoparticles in tumor cells by increasing absorption in the NIR where tissues present low NIR region radiation absorption, after irradiation. Kahalalide F analogue has been conjugated with AuNPs to enhance the delivery of Kahalalide F to tumoral cells by increasing its cytotoxic effect. Cellular uptake of conjugates has been studied into HeLa cells. Two different sizes of AuNPs, i.e., 20 and 40 nm (AuNP-20, AuNP-40, respectively) have been made to determine the influence of nanoparticle size on the penetration and cytotoxicity of the conjugates. As expected, the accumulation of AuNP-40 is higher than AuNP-20. AuNP-40 conjugates also show higher cytotoxicity with respect to the free peptides. This enhancement could be due to better cell uptake of the former conjugate.

Liposomes can be used as a 'Trojan Horse' to deliver small (1.4 nm) AuNPs into tumor cells [100]. It is revealed that the liposomal approach provides a thousand-fold enhancement in the cellular uptake of the small AuNPs. Real-time intracellular tracking of the AuNP-liposomes reveals an average speed of 12.48 ± 3.12 $\mu\text{m/hr}$ for their intracellular transport.

Conclusion and Future Outlook

In this chapter, I have focused on different vehicles for internalization of gold nanoparticles inside cells and applications of internalized particles in nanomedicine. Several ligands such as peptides, proteins and small molecules have been successfully used for specific delivery of AuNPs. It is now possible to control the size of gold nanoparticles at nanometer resolution. Additionally, the biocompatibility and photo-optical distinctiveness of gold nanoparticles are now proven to be powerful in diagnostic applications.

However, a very important issue in the successful implementation of the promised applications of AuNPs is toxicity. There are many reports available on non-toxicity of AuNPs. AuNPs emerge much less toxic than other types of nanoparticles. Some of the ligands used for AuNPs-conjugation are moderately toxic. Many biocompatible ligands including thiolated PEGs are non-toxic. Another issue is the poor reproducibility and wide range of size distribution of nanoparticle product synthesized from wet chemical methods. Improved synthetic methodologies that can lead to true monodispersity and satisfactory reproducibility of nanoparticles with well-controlled shape remain to be developed. Recent reports show that AuNPs –based therapeutic agents could overcome the barriers presented by the human immune systems to achieve delivery at diseased sites without effecting healthy tissues. In conclusion, AuNPs are easily bioconjugable and very promising for imaging, diagnostics and therapy biomedical applications for cancer and a number of other diseases for human.

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Chapter 12

A COMMENTARY ON NEURAL TISSUE ENGINEERING IN THE CENTRAL NERVOUS SYSTEM – INTERFACING A LESION

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ABSTRACT

Damage to the Central Nervous System (CNS) from disease or injury remains a serious problem that affects millions of individuals worldwide. Regeneration across a lesion in the adult mammalian CNS is autonomically inhibited, thereby presenting substantial challenges for clinical treatment strategies. Current methods employed such as autografts, xenografts and allografts have limited success due to the complex nature of CNS injury pathophysiology. As a result, there is no effective treatment available to establish repair of damaged neurones within the CNS, with patients typically being administered a combination of drugs and rehabilitation in attempts to maintain and restore some functionality. Recent research has explored the potential of synthetic scaffolds to fabricate biomimetic regenerative microenvironments for nerves as an alternative treatment option. This commentary addresses the importance of scaffold design in enhancing the regeneration capacity of the adult CNS; focusing on past innovative strategies and current problems. Future trends towards fabricating scaffolds to achieve superior regenerative outcomes will also be highlighted.

INTRODUCTION

The injured brain or spinal cord has limited capacity to recover because axonal regeneration across a lesion site fails to occur resulting in permanent functional disabilities to the patient. This problem persists as currently treatments are limited to drug and/or rehabilitation therapies.

The pathology of CNS injury is not only dictated by the initial tissue insult, but also by secondary injuries encompassing ischemia, anoxia, free-radical formation, excitotoxicity and

suchlike, which can occur for several days to weeks after the initial injury [1]. Following traumatic injury to the CNS, a common misconception is that secondary injury actively inhibits nerve regeneration. However post traumatic injury studies of the spinal cord and cortex have shown the existence of axonal sprouting within a few millimeters adjacent to the injury (ie. within the penumbra) [2-8]. Therefore, the current neurological problem is not to encourage the sprouting of endogenous neurites post injury, but rather to persuade them into and across the lesion site to facilitate the reformation of synapses. Therefore it is important to understand the inflammatory response after injury, in particular the timing of the microglia, astrocyte and macrophage activation in an attempt to control and manipulate their response to encourage a primarily cytotoxic role.

INFLAMMATION

Following injury to the caudate putamen; astrocytes, microglia and macrophages are activated for approximately 1 week before their numbers begin to decline [9, 10]. They accumulate within the penumbra area, typically within the first 3 millimeters from the injury site. These cells are responsible for the degree of axonal sprouting that ensues in this area, whilst also influencing the tendency of axons to enter the lesion.

Microglia and macrophages are capable of sensing changes within the extracellular environment and are phagocytotic [11, 12]. The response of these cells takes place over two phases, which are often classed as their cytotoxic and cytotropic roles of repair. Initially they attempt to maintain tissue integrity through the elimination of damaged tissue and foreign matter by phagocytosis. It is this response that is referred to as cytotoxic, as often in the process they damage viable neighboring neural tissue [11, 12]. The second stage of repair is where these cells facilitate axonal sprouting. This occurs via several processes, including the secretion of anti-inflammatory cytokines and the stripping of damaged axonal branches [13]. Arguably their most cytotropic role is the expression of different neurotrophic factors. Microglia and macrophages are responsible for the expression of brain derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) in the penumbra, respectively [14]. Reducing the secretion of these neurotrophic factors inhibits axonal sprouting; supporting the notion that axonal sprouting within the penumbra is dependent on the number of activated microglia and macrophages present [14, 15].

However, despite the presence of axonal sprouting in the penumbra following traumatic injury, there is a lack of axonal infiltration into the lesion. This is most likely due to the activation of astrocytes. Following CNS injury, astrocytes morph into what is termed "reactive astrocytes", appearing larger (hypertrophic) with longer and more highly branched processes [16, 17]. These cells proliferate and migrate within the injury site in an attempt to maintain tissue integrity by isolating the lesion area. As a result they distort the local CNS architecture and produce glial scarring, a role predominantly carried out by fibrous astrocytes [18]. In addition to this mechanical barrier at the site of the injury, reactive astrocytes can also produce a biochemical barrier. Specific molecular guidance molecules [19] are expressed by reactive astrocytes in response to an injury, which repel neurites and prevent reinnervation through the lesion and regeneration of axons [19]. Despite this, they also contribute a cytotropic component to repair during inflammation. Although astrocytes appear to have a

detrimental affect following an injury, their control of the normal CNS environment is essential. These cells are responsible for metabolically supporting neurones by providing nutrients such as glucose. They are also critical to the maintenance of the blood-brain-barrier as they biochemically support endothelial cells. Astrocytes undertake many other functions *in vivo* some of which are not fully understood or even determined. However some of their roles are known to include: the expression and release of membrane transporters that are critical to many neurotransmitters [20], the expression of potassium channels to control ion levels within the CNS; they have been shown to influence synaptic transmission through quick morphological changes [21] and are involved in the modulation of neuronal blood flow [22], and they support oligodendrocytes through promoting neurones to release ATP which is an important oligodendrocyte stimuli for the production of myelin [23].

In-order to achieve axonal infiltration within a lesion it is important to control the extent and temporal component of the microglia, macrophage and astrocyte response and their cytotoxic and cytotrophic contributions. Attempts to avoid such levels of control have been made by bridging the lesion using nerve grafts such as autografts, allografts and xenografts. Such strategies have had varying degrees of success after being grafted into the spinal cord and are not viable due to limited supply in the case of autografts and the need for immunosuppressive therapy in the case of allografts and xenografts [24]. Therefore neural tissue engineering strategies have investigated the introduction of scaffolds into the lesion, to attempt to control the inflammation and provide a microenvironment that is conducive to axonal elongation and maintenance [25, 26].

THE FORMATION OF THE ECM WITHIN THE CNS AND ROLE FOLLOWING CNS INJURY

Here a brief discussion of the formation of the extracellular matrix (ECM), which occurs during development is warranted to aid in the following commentary that will address the importance of scaffold design in encouraging axonal sprouting in the adult CNS, ie. their ability to provide a conducive microenvironment. The lattice-like structure of the multiple components of the ECM is often referred to as the perineuronal net (PNN), which encapsulates the synapses in the parenchyma of the brain and spinal cord. This network is essential to maintain ion homeostasis, provide neuroprotection and prevent unwanted plasticity.

In the developing CNS, neural precursor cell differentiation is the predominant source of neuronal and glial cells [27, 28]. Along with the initial migrating neural precursor cells, radial glia is one of the earliest cellular phenotypes present in the developing CNS. Radial glia provide a supportive matrix, which facilitates the guidance of neural precursor cells and immature neurones during developmental migration in the cerebral cortex [29, 30]. However, until recently the influence of radial glia during development was believed to only offer structural support, as it was recognised that growing tissue was oriented orthogonally with these cells [30]. It is also acknowledged that fibronectin (FN) and chondroitin sulfate proteoglycans (CSPG), are critical ECM components influencing neuronal proliferation, migration, layer formation (in the cortex) and axonal elongation [31]. The biomolecular support offered by radial glia has now also been acknowledged, with their capacity to produce

and align the critical ECM component FN *in vivo* and *in vitro* being determined [30]. Through integrin receptor interaction with FN on the radial glia cell surface, the proliferation, migration, and axonal elongation of migrating precursor cells and neurones in the developing CNS is influenced and controlled. Therefore, radial glia are arguably the most essential cellular phenotype for the formation of the ECM during development. They offer a framework to physical and biochemically support and guide immature neurones as they migrate, whilst also expressing ECM molecules essential to control neuronal migration and axonal elongation.

After the transformation of radial glia postnatally, left over material from this process constitutes, in part, some of the ECM within the adult CNS. Knowledge of the developing CNS is a critical foundation when designing a scaffold to be introduced into a neural lesion. One approach for attempting to regenerate tissue within the adult CNS is to focus on the process of development, where it may be useful to mimic some of the physical and biochemical features of the radial glia. A further commentary will be provided on this point whilst discussing scaffold design.

Interestingly, CSPGs are the major inhibitory component of glial scars [32]. Elevated levels of CSPGs, which are the major component of PNNs, can halt regeneration and inhibits the growth of axons through the lesioned/damaged region of the CNS. Furthermore, this CSPG-mediated inhibition has been linked to the activation of several signaling pathways. It is now well established that the inhibitory effect of the sulfated proteoglycans can be improved by administration of enzyme chondroitinase ABC (ChABC) both *in vivo* and *in vitro* [33, 34]. More recently, it was shown that administration of ChABC into the cerebral cortex could enhance plasticity [35], which would be a powerful tool to exploit in neuronal repair.

In summary, what all these studies do demonstrate is the necessity for combinatorial strategies in the functional regeneration of the lesioned CNS, and that the synergy of biochemistry and tissue engineering will be essential in future developments.

FEATURES AND CHALLENGES OF SCAFFOLDS FOR CNS REGENERATION

An ideal material to fabricate a neural tissue engineering scaffold should not demonstrate cytotoxicity or immunogenicity or an unresolved foreign body reaction. In addition, the mechanical properties should be easily tunable to match that of the surrounding tissue. This is designed to avoid structural collapse of the penumbra area into the lesion if the modulus is too small, or micro tearing of the tissue at the lesion/penumbra interface if it is too large. These requirements are critical to the success of scaffolds implanted within a CNS lesion. Much research has focused on fabricating and testing biocompatible and compliance matched biomaterials for this application [36-39]. However, an ideal scaffold for repair within the CNS would also offer several other properties similar to that of the radial glia during development. They should have the capacity to:

- Offer structural similarity, facilitating neuronal migration and axonal elongation utilising the topographical features of the scaffold.

- Present ECM molecules, growth factors, cell adhesion and signaling molecules at specific times during the regenerative process.
- Have the capacity to be used as a vessel for cell encapsulation to introduce replacement cells and/or support cells into the lesion, whilst facilitating their viability and migration, if this is the desired strategy.

Such a scaffold would mimic some of the attributes of radial glia during development, by providing a physical and biochemical framework for migration and ultimately regeneration. We believe that such an approach will generate successful outcomes in the field, as post injury regeneration does occur in the penumbra. Hence, the challenge lies in harnessing this native reaction to provide reinnervation within the lesion. Therefore the presence of radial glia like support, based on the physical and biochemical architecture of the CNS in development, may facilitate such migration.

In the past much research has focused on producing biodegradable scaffolds for CNS regeneration. There are two reasons for this; first it was assumed that this would eliminate the need for secondary surgery to remove the implant [24]; and second, it was assumed that when the scaffold degrades the cells will secrete molecules capable of reforming the native ECM [40, 41]. However a permanent implant, that is biofunctionalised, integrated and compliance matched to the surround tissue, may be a more suitable scaffold, particularly for large lesions.

As radial glia are by-large responsible for the fabrication of the ECM and they are not present in the adult CNS, it will not be possible for cells to reform their native ECM to physically and biochemically support themselves across large lesions. Therefore, scaffold biodegradation may reform a cavity, which would ultimately facilitate tissue collapse and scar formation. However, a permanent implant that mimics some of the physical and biochemical attributes of the radial glia during development may encourage reinnervation whilst offering long-term structural support and protection to the migrating neurones (i.e. migration from ventral portion in the cortex) and their processes, within and beyond the lesion area.

CURRENT SCAFFOLD DESIGN

Scaffolding materials for implantation into a lesion within the CNS have been produced from a variety of different methods. Most of this research has been focused on hydrogels and more recently electrospun polymer nanofibres and self assembling scaffolds.

Natural and synthetic hydrogels have shown considerable promise because various types can be injected in a minimally invasive manner; their modulus can be easily tailored; and they can be readily functionalised with biomolecules. Adhesive peptides such as RGD are commonly incorporated within synthetic hydrogels to facilitate superior integrin interaction, thus resulting in superior axonal in-growth within the spinal cord [25]. Similarly, injectable and thermally gelling hydrogels have been optimised to repair damaged neural pathways within the brain [42, 43]. One of the main disadvantages of hydrogels is that such materials cannot provide directional cues for regeneration due to their isotropic architecture. For this reason it is often required to blend neurotrophic factors within the gel or create chemotactic gradients [44], which has not been successful over large distances.

Electrospun polymer nanofibres have also shown considerable promise because they provide similarity to the nanodimensions of the cellular microenvironment within the brain tissue. They essentially mimic some of the physical features of the ECM [45, 46] produced by radial glia. Electrospun scaffolds provide contact guidance cues for neurite outgrowth [47, 48] growing on top of the fibrous structure similar to their migration and perpendicular elongation during development. Axonal orientation has been analyzed on poly(dimethyl siloxane) surfaces with grooves generated using lithography. Both parallel and perpendicular contact guidance was evident, which was dependant on the width and depths of the channels [49]. Further to this, partially aligned electrospun scaffolds implanted into the caudate putamen of the adult rat brain supported perpendicular contact guidance of the endogenous neurites [26]. We have recently tethered BDNF to electrospun fibres in an attempt to control the adhesion, proliferation and differentiation of neural stem cells and have demonstrated an ability to achieve this whilst also directing the differentiation of these cells [50]. However, a major limitation of using electrospun scaffolds across a lesion is that their nature prevents intimate contact with the edge of the penumbra, which in effect still leaves a lesion although somewhat smaller.

Biologically inspired self assembling peptide scaffolds have also been utilised for CNS regeneration, due to their fibrous morphology and ease of injection (i.e. they are a liquid which self assemble *in vivo*). Self assembling peptide scaffolds also have the ability to incorporate signaling sequences. For instance, high densities of the laminin epitope IKVAV have been assembled in the mouse spinal cord which reduced astrogliosis and cell death while increasing the number of oligodendrocytes in the injury site. They also promoted the regeneration of both descending motor and ascending sensory fibres [51]. However, a limitation with these scaffolds is their small pore size of approximately 5-200 nm [52], limiting cell migration or neurite outgrowth until degradation creates room for these events.

PERSPECTIVE

Scaffolds designed for CNS repair should have the capacity to be implanted within a lesion whilst mimicking some of the critical features of the cellular microenvironment present in the developing or adult CNS that will optimise repair. Most injectable scaffolds used for this purpose have been hydrogels, but problems exist regarding their usefulness as discussed previously. The fabrication of a scaffold that simulates some features of the developing ECM is important to support the migration of endogenous neural precursor cells and neurones; and/or maintain implanted stem cells depending on the employed tissue engineering strategy. The scaffold must also facilitate the migration of neurones and guide their processes, allowing them to enter the lesion and reform synapses, whilst also organising the position of support cells from the penumbra such as glia.

The use of a composite scaffold may be a possibility as the requirements mentioned above are independent. For example, electrospun nanofibres with layered architectures that mimic the six-layered structure within the mammalian cortex, could be fabricated to consist of aligned and randomly orientated nanofibres (providing protection and an appropriate microenvironment). Similarly, a composite scaffold, consisting of aligned, layered and/or short nanofibres within a tube filled with a hydrogel has merit as this scaffold would

encourage more intimate contact with the edge of the penumbra than electrospun polymer fibres and offer superior structural support. Such a strategy could also incorporate biomolecular cues, either as soluble molecules or by tethering to either the hydrogel or polymer fibre to limit the inflammatory reaction with the penumbra area. There is also an option for different molecules to be incorporated within each of the scaffolds offering more complex biomolecular support. Such a multifaceted strategy is designed to control the inflammatory response within the penumbra to encourage sprouting axons in this area to infiltrate the lesion. Iterative optimisation of such a strategy may simulate some of the physical and biochemical processes that occur during development and move closer towards meeting some of the demands for neuronal migration and process elongation within a lesion.

CONCLUSION

While the long term aim of neural tissue engineering is the regeneration across, and minimizing the size of the lesion, there is much work to be conducted in regard to designing an appropriate scaffold that can mimic the highly complex temporal and spatial evolution of the developing CNS before this aim can be realised. In-order to design such as scaffold it is critical to understand the molecular, cellular and structural processes that influence and control the migration of neurones and elongation of their neurites during development. Insight into the manner by which neurones form synapses and generate the interconnected architecture of the CNS, as well as the timing of such events must also be considered. We believe that a combination strategy, incorporating several different scaffold forms that have been independently functionalised with different biomolecules may be useful. Further to this, due to the absence of radial glia within the adult mammalian CNS post development, which to a large extent facilitates the formation of the ECM, we believe that it is not necessary to have a degradable scaffold when bridging a large lesion within the CNS. A permanent scaffold that mimics the native cellular microenvironment, is compliance matched to the endogenous tissue, and encourages reinnervation may be appropriate to support neurone migration and neurite infiltration within the lesion, as long as this does not potentiate neurotoxic processes (eg. microglial activation).

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